## REMARKS/ARGUMENTS

Claims 2, 5-6, and 8-12, 14-38, 53 and 54 were pending in the subject application. Applicants respectfully note that claims 2, 8, 9, 11, 12 and 53 have been amended, and claims 55-57 have been added. This amendment does not involve any issue of new matter. Support for the amendment to claims 2 and 53 may be found in the originally filed specification, *inter alia*, on page 6, lines 22-26, page 11, lines 5-15, page 9, lines 9-13, pages 15-16, examples 1-2, and page 9, lines 5-13. Support for added claims may be found originally filed specification, *inter alia*, as follows: claim 55, page 6, lines 26-35, and claims 56-57, on page 3, lines 24-28. Applicants respectfully request entry of the subject amendment such that claims 2, 5-6, 8, 9, 11, 12, 14-38 and 53-57 will be pending.

## Claim Rejections - 35 U.S.C. §103

The Office Action rejects claims 2, 5, 6, 8-12, 14, 23, 24, 26, 27, 35-38, 53 and 54 under 35 U.S.C. 103(a) as being allegedly unpatentable over Kelly (U) in view of Kuberasampath (AG) and Lefer(V). The Office Action further rejects claims 2, 15-20 and 53 as being unpatentable over Kelly (U) in view of Kuberasampath (AG) and Lefer(V), and further in view of Anderson(U) and Brady (W).

The Office Action alleges on page 3 that "Kelly discloses that mutant mice genetically deficient in ICAM-1 are protected from acute renal ischemic injury as judged by serum creatine, renal histology and animal survival" and that "the protection afforded by knockout of the ICAM-1 gene is due to prevention of leukocyte accumulation in the kidney." The Office Action further alleges that "the data of Kelly suggests that agents designed to block leukocyte-endothelial interactions mediated via ICAM-1 may be therapeutically effective in the prevention and treatment of acute renal failure." The Office Action does concede that Kelly fails to teach administering OP-1 to a mammal afflicted with acute renal failure."

The Office Action further alleges that Kuberasampath teaches that morphogens, such as OP-1, may be used to treat inflammation and to reduce the tissue-destructive effects of inflammation,

such as inflammation caused by ischemic reperfusion injury following renal artery occlusion.

The Office Action further alleges that Lefer teaches that hOP-1 exhibits significant anti-adherent actions on PMNs.

The Office Action then concludes, on pages 7-8, that "(i)t would have been obvious to one of ordinary skill in the art at the time of Applicant's invention to administer an agent designed to block leukocyte-endothelial interactions to a mammal afflicted with ARF, as taught by Kelly, and to modify that teaching by administering OP-1, as taught by Kuberasampath, with a reasonable expectation of success." On this basis, the Office Action rejects claims 2 and 53, from which all other claims depend.

The Office Action further rejects claim 20 on the basis of Brady, which allegedly teaches that prolonged hypoperfusion may lead to intrinsic renal azotemia, and claims 15-19 on the basis of Anderson, which allegedly teaches that the serial determination of BUN and creatine levels.

In response, applicants first note that claims 2 and 53 have been amended to clarify the claimed invention. Applicants submit that claims 2 and 53 recite methods "of effecting an improvement in a standard marker of renal function in a mammal afflicted with acute renal failure" and not methods "of treating inflammation in a mammal afflicted with acute renal failure." Applicants further submit that the claimed methods, by improving a standard marker of renal function, would be useful in delaying the need for, or reduce the frequency of, dialysis treatments (as set forth on page 3, lines 24-28 of the specification) since such dialysis treatment may artificially provide renal filtering function. Applicants note that they have added new claims 56 and 57.

MPEP 706.02(j) sets forth three basic criteria needed to establish a *prima facie* case of obviousness: 1) the prior art references must teach or suggest all the claim limitations; 2) some motivation or suggestion, either found in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to combine or modify the references must be present;

and 3) a reasonable expectation of success is required.

Since the Office Action fails to set forth at least criteria (1) and (3), it fails to establish a *prima facie* case of obviousness, as described in more detail below.

**A. Lack of a Reasonable Expectation of Success**

The combined teachings of Kelly, Kuberasampath, and Lefer fail to provide a reasonable expectation of success for effecting an improvement in a standard marker of renal function in a mammal afflicted with acute renal failure by administering an OP/BMP renal therapeutic agent.

The Office Action assumes that if an agent is known to reduce inflammation, then one skilled in the art would reasonably expect that agent to be effective in treating acute renal failure. Based on this central assumption, the Office Action concludes that since OP-1 is allegedly effective in treating inflammation, then one skilled in the art would reasonably expect OP-1 to be effective in treating acute renal failure. However, this argument fails because at the time the subject application was filed, anti-inflammatory agents, and in particular inflammatory agents which decrease leukocyte adhesiveness and/or ICAM-1 expression, were known to decrease renal function, or even to cause outright renal failure, upon their administration to a mammal. Accordingly, one skilled on the art would have expected that administration of the alleged antiinflammatory OP-1 to a mammal afflicted with acute renal failure would have aggravated, not improved, renal function in the mammal *i.e.* one skilled in the art would not have had a reasonable expectation that OP-1 would be effective in treating acute renal failure, let alone be effective in effecting an improvement in a standard marker of renal function as recited in claims 2 and 53, from which all other claims depend.

(1) Transforming Growth Factor Beta 1 (TGF-β1)

TGF-β1 shares 35% amino acid identity with the seven-cysteine domain of OP-1 **(Exhibit A)**. Both OP-1 and TGF-β1 are members of the TGF-β superfamily and both bind to cell-surface receptors which activate intracellular signaling via the SMAD proteins.

At the time the subject application was filed, TGF-β1 was well-known as an antiinflammatory molecule. For example, Kulkarni AB et al., *Am J Pathol.* 1995;146(1):264-75 **(Exhibit B)**, describes massive inflammation in mice in the absence of TGF-β1. The abstract states as follows:

> Approximately 40% of transforming growth factor-beta 1 null (knockout) mice generated in our laboratory develop normally to term, but 60% die in utero. The animals appear normal during the first 2 weeks of life but develop a rapid wasting syndrome and die by 3 to 4 weeks of age. All of the knockout mice have a multifocal inflammatory disease in many tissues... In the absence of any pathogens, this massive inflammatory disease, together with overexpression of major histocompatibility complex class I and II proteins and overproduction of immunoglobulins by lymphocytes, offers circumstantial evidence for an autoimmune etiology. (Emphasis added).

Similarly, Gamble JR, *J Immunol.* 1993;150(10):4494-503 (**Exhibit C**), states in the abstract that "Transforming growth factor-beta (TGF-beta), a pleiotropic cytokine that is elaborated in the active form upon co-culture of endothelial cells and pericytes or smooth muscle cells, has been shown to decrease the adhesiveness of endothelial cells for neutrophils, lymphocytes, and tumor cells" (emphasis added). The ability of TGF-β1 to decrease neutrophil adhesiveness is particularly relevant, because according to the office action, Lefer teaches that OP-1 reduces neutrophil adhesion, and that this property provides a high expectation of success for the effectiveness of OP-1 in treating acute renal failure.

However, in spite of its anti-inflammatory and neutrophil adhesion-inhibiting properties, TGFB1 was known, at the time the subject application was filed, to cause and/or aggravate renal disease. For example, Ketteler M. et al. *Curr Opin Nephrol Hypertens.* 1994; 3(4):446-52 (**Exhibit D**), states in the abstract that "(r)ecent studies show that TGF-beta overexpression in experimental and human kidney diseases leads to progressive glomerular and tubulointerstitial scarring and renal failure," and that "(n)ew therapies may prevent progressive fibrosis in chronic kidney disease by suppressing the action of TGF-beta."

Similarly, Border WA et al. *Nature.* 1990 26; 346(6282):371-4 (**Exhibit E**) provides direct experimental evidence of the role of TGFβ1 in renal disease in a mammal. The abstract states as follows:

> We have used an animal model of acute mesangial proliferative glomerulonephritis to show that this disease is associated with increased production and activity of transforming growth factor beta 1 (TGF-beta 1), an inducer of extracellular matrix production. Here we report that administration of anti-TGF-beta 1 at the time of induction of the glomerular disease suppresses the increased production of extracellular matrix and dramatically attenuates histological manifestations of the disease. These results provide direct evidence for a causal role of TGF-beta 1 in the pathogenesis of the experimental disease and suggest a new approach to the therapy of glomerulonephritis. Accordingly, one skilled in the art would have been taught against administering TGFβ1 to a mammal afflicted with acute renal failure.

In further support of a causative role of TGF-β1 in renal disease, applicants present Border WA. *Curr Opin Nephrol Hypertens.* 1994; 3(1):54-8 (**Exhibit F**). The abstract recites as follows:

> In a model of acute mesangial proliferative glomerulonephritis, it was shown that overproduction of TGF-beta is the cause of pathologic matrix accumulation in the nephritic glomeruli. TGF-beta acted to increase matrix production, inhibit matrix degradation, and modulate matrix receptors in the glomerulonephritic rats. Elevated expression of TGF-beta was also found in other experimental glomerular diseases, including diabetic nephropathy. Studies of humans with glomerulonephritis and diabetic nephropathy also strongly implicated TGF-beta in the pathogenesis of glomerular matrix build-up.

Accordingly, the above references demonstrate that one skilled in the art would not have expected that the antiinflammatory growth factor TGF-β1 would be effective in treating acute renal failure, let alone in effecting an improvement in a standard marker of renal function in a mammal afflicted with acute renal failure. On the contrary, one skilled in the art would expect TGF-β1 administration to decrease renal function in a mammal afflicted with acute renal failure. Given (a) the significant structural similarity between OP-1 and TGF-β1, (b) their functional similarities of signaling through the SMAD proteins, (c) their shared anti-inflammatory properties, and (d) their shared property of inhibiting neutrophil adhesion, one skilled in the art would have expected OP-1, like TGF-β1, to decrease renal function in a mammal afflicted with acute renal failure, and therefore would have had no reasonable expectation of success in improving renal function in a mammal

afflicted with acute renal failure by administering a OP/BMP renal therapeutic agent as recited in claims 2 and 53. Accordingly, the Office Action has failed to make a case of *prima facie* obviousness.

(2) Cyclosporin A (CsA)

Cyclosporin A (CsA), a hydrophobic cyclic peptide isolated from *Tolypocladium inflatum*, was known at the time the subject application was filed to be an anti-inflammatory agent, and in particular, a suppressor of ICAM-1 expression. For example, Oran A. et al.*Br J Dermatol.* 1997;136(4):519-26 (**Exhibit G, Abstract only**), states in the abstract that "CsA exhibits a wide range of anti-inflammatory effects including reduction of ICAM-1 expression and mast cell numbers."

Furthermore, Frishberg Y et al. *Kidney Int.* 1996; 50(1):45-53 (**Exhibit H**), found that cyclosporin even reduces expression of ICAM-1 in renal cells. The abstract states that "(w)e have found that CsA has a concentration dependent effect on the expression of both ICAM-1 mRNA and gene product on renal tubular cells." In addition, not only does CsA suppress ICAM-1 expression it does so through a different mechanism as TGF-B1, as the abstract further states that "TGF-beta 1 has similar effects on ICAM-1 and LFA-1 expression as high dose CsA, but the CsA effects are not mediated through induced TGF-beta 1 expression." Therefore, yet another class of anti-inflammatory agents, represented by cyclosporin and acting through a different mechanism as TGF-β1, were known to reduce ICAM levels at the time the subject application was filed.

Although CsA is a well-established anti-inflammatory and anti-ICAM-1 agent, key properties of OP-1 alleged in the Office Action to render obvious the use of OP-1 for treating acute renal failure, CsA was also known at the time the invention was made to reduce renal function. For example, Wissmann C. et al. *J Am Soc Nephrol.* 1996; 7(12):2677-81 (**Exhibit I**), states in the abstract that "Cyclosporine A causes an acute reduction in GFR." (GFR stands for Glomerular Filtration Rate). In the study described in Wissmann, kidney transplant patients were treated with or without cyclosporin. Wissmann notes that "In these patients, a mean (+/- SD) maximum

cyclosporine-induced increase in serum creatinine concentration of 13 +/- 11% ($P < 0.001$) and in serum BUN of 27 +/- 33% ($P < 0.01$), together with a decline in the fractional uric acid excretion of 51 +/- 89% ($P < 0.02$) were observed." Thus, administration of cyclosporin to kidney transplant recipients resulted in a significant reduction in a two standard markers of renal function i.e. serum creatine and BUN levels, and not in an increase.

Accordingly, one skilled in the art would have expected the administration of the anti-inflammatory anti-ICAM-1 OP-1 polypeptide, similar to the known effects of the anti-inflammatory agent CsA, would reduce renal function, and not to increase in renal function. One skilled in the art would have had no reasonable expectation of success for effecting an improvement in a standard marker of renal function in a mammal afflicted with acute renal failure by administering OP-1. In fact, the CsA references cited above would teach away from using the antiinflammatory agent OP-1 to treat acute renal failure. Accordingly, the Office Action has failed to make a case of *prima facie* obviousness.

(3) Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

NSAIDs, widely used anti-inflammatory agents, include salicylates (aspirin), propionic acids (ibuprofen), indolacetic acids (indomethacin) and anthranilic acids. In addition to their well-documented anti-inflammatory properties, NSAIDs were also known to reduce neutrophil adhesion, a key property of OP-1 that the Office Action alleges renders obvious its use in treating acute renal failure. For example, Diaz-Gonzalez F. *J Clin Invest.* 1995; 95(4):1756-65 (**Exhibit J**), states in the abstract that "(s)ome nonsteroidal antiinflammatory drugs (NSAIDs), such as indomethacin, diclofenac, ketoprofen, and aspirin, but not steroids, strongly inhibited the neutrophil-endothelial cell attachment." Diaz further teaches that "(t)hese results suggest that NSAIDs exert a specific action on adhesion receptor expression in neutrophils, which might account, at least in part, for the antiinflammatory activities of NSAIDs."

Accordingly, NSAIDs shared the two key features with OP-1, anti-inflammatory properties

and anti-neutrophil adherence properties, that the Office Action alleges renders obvious the use of OP-1 to treat acute renal failure. However, NSAIDs were know to reduce renal function, and even to contribute to kidney failure, particularly in patients suffering from preexisting impairments in kidney function. For example, the review article Whelton et al. (1991) *Clin Pharmacol.* 31(7):588-98 (**Exhibit K**), teaches that "approximately 1-5% of people who are exposed to a nonsteroidal anti-inflammatory drug (NSAID) will manifest one of a variety of renal function abnormalities ... Renal abnormalities include fluid and electrolyte disturbances, acute deterioration of renal function, nephritic syndrome with interstitial nephritis, and papillary necrosis" (Page 588, columns 1-2). Furthermore, Whelton et al. teaches that "from the clinical point of view, the most worrisome renal side effect of NSAIDs is hemodynamically mediated acute renal failure, which occurs in individuals with pre-existing reduced renal blood perfusion" (Page 588, column 2) (emphasis added).

The review article Bennet et al. (1996) *Am J Kidney Dis* 1996 28 (1 Suppl 1):S56-62 (**Exhibit L**) provides recommendations based on a critical literature survey. Bennet concludes that while the use of NSAIDs in the general population is safe and effective when used in therapeutic dosages for a limited period of time, "patients with pre-existing risk factors are susceptible to potentially life-threatening toxicities, including acute renal failure (ARF) and serious fluid and electrolyte disorders" (page S-61, column 1).

Similarly, another review article by Murray et al., (1997) *Prog Drug Res.* 49:155-71 (**Exhibit M, abstract only**), warns of the risk of administering anti-inflammatory agents to patients with abnormal kidney function in the abstract: "Among persons with normal renal function, who have no other risk factors (dehydration) for an acute hemodynamic effect, there is no risk. However, NSAID administration to susceptible persons may cause decrements in renal plasma flow and glomerular filtration rate within hours" (emphasis added).

Accordingly, one skilled in the art would have expected that administration of the anti-inflammatory OP-1 polypeptide, based on the anti-inflammatory and neutrophil adhesion-inhibiting properties that its shares with NSAIDs, would reduce, rather than increase, renal function. One

skilled in the art would have had no reasonable expectation of success for increasing renal function in a mammal afflicted with acute renal failure by administering the anti-inflammatory OP-1. In fact, the NSAIDs references cited above would teach away from using the anti-inflammatory agent OP-1 to treat acute renal failure. Accordingly, the Office Action has failed to make a case of *prima facie* obviousness.

**B. References Fail to Teach or Suggest all the Claim Limitations**

The combined teachings of Kelly, Kuberasampath, and Lefer also fail to teach or suggest all the elements of claims 2 and 53, and therefore cannot render these claims obvious. Applicants submit that claims 2 and 53 recite a "method of effecting an improvement in a standard marker of renal function in said mammal" (emphasis added).

By contrast, the combined teachings of these reference teach, at best, the administration of a morphogen to treat inflammation in a mammal afflicted with acute renal failure. The Office Action incorrectly assumes that treating renal inflammation is synonymous with improving renal function. The Office Action also assumes, without providing evidence, that treating renal inflammation is sufficient to improve renal function. Applicants submit that the pending claims do not recite a methods of treating inflammation, but rather methods "of effecting an improvement in a standard marker of renal function in said mammal." Since the combination of the cited references fails to teach or suggest at least this element of the claims, it fails to render the invention obvious. Applicants further note that the combination of Kelly, Kuberasampath, Lefer, Anderson and Brady likewise fails to teach or suggest every element of the claims and thus does not render obvious any of the pending claims.

**C. Teaching Away from the Claimed Invention**

The references cited by the Examiner combined with the references cited above by applicants not only fail to provide a reasonable expectation of success, but also teach away from the claimed invention.

Pursuant to MPEP 2144.05," [a] prima facie case of obviousness may also be rebutted by showing that the art, in any material respect, teaches away from the claimed invention." In addition, MPEP 2145 states that "[a] prior art reference that 'teaches away' from the claimed invention is a significant factor to be considered in determining obviousness."

The references cited by applicants clearly teach away from using anti-inflammatory agents, including members of the TGF-β superfamily such as OP-1, to improve a marker of renal function in a mammal afflicted with acute renal failure. In *re Gurley*, 27 F.3d 551, 553, 31 USPQ2d 1130, 1131 (Fed. Cir. 1994) states that "[a] reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the applicant." One of ordinary skill in the art reading Exhibits A-M would be both (i) discouraged from using an anti-inflammatory agent like OP-1 to increase renal function, especially in a mammal afflicted with acute renal failure, since anti-inflammatory agents were known to decrease renal function; and (ii) led away from the path of using anti-inflammatory morphogens and into other paths, such as as that of using agents with no anti-inflammatory properties or of treating subjects with dialysis.

By teaching away from the claimed invention, the references cited above in combination with the references cited by the examiner including Kuberasampath and Lefer rebutt the Examiner's alleged case of prima facie obviousness. Applicants respectfully request reconsideration and withdrawal of this ground of rejection.

## Conclusions

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue.

Applicant believes no fee is due with this response in addition to the $110 fee for the one-month extension of time. However, if an additional fee is due, please charge our Deposit Account No. 18-1945, under Order No. JJJ-P01-514 from which the undersigned is authorized to draw.

Dated: November 12, 2004

Respectfully submitted,

By _____

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          CKVHELYVSFRKDLGWQDWIIAPEGYAANFCLGECAFPLNIYSLATNHAI
                   10        20        30        40        50
TGFB1 C CCVRQLYIDFRKDLGWK-WIHEPKGYHANFCLGPCPY---IWSLDTQYSK 339
OP1 Cys CKKHELYVSFR-DLGWQDWIIAPEGYAAYYCEGECAFPLNSYMNATNHAI 49

          VLALVNQINPGAVAAPCCAPTALEALSVVYFVGSSNVIVEQLSNMVVRAC
                   60        70        80        90       100
TGFB1 C VLALYNQHNPGASAAPCCVPQALEPLPIVYYVGR-KPKVEQLSNMIVRSC 388
OP1 Cys VQTLVHFINPETVPKPCCAPTQLNAISVLYFDDSSNVILKKYRNMVVRAC 99

          GCS

TGFB1 C KCS                                                391
OP1 Cys GCH                                                102
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Sequence pair distances of Untitled ClustalW (Slow/Accurate, Gonnet)
Friday, November 12, 2004 1:34 PM

Percent Identity

Divergence

| | 1 | 2 | |
|---|---|---|---|
| 1 | | 34.7 | 1 |
| 2 | 127.6 | | 2 |
| | 1 | 2 | |

TGFB1 Cys Domain
OP1 Cys Domain

American Journal of Pathology, Vol. 146, No. 1, January 1995




# Animal Model

## Transforming Growth Factor-β1 Null Mice

### An Animal Model for Inflammatory Disorders

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***Approximately 40% of transforming growth factor-β1 null (knockout) mice generated in our laboratory develop normally to term, but 60% die in utero. The animals appear normal during the first 2 weeks of life but develop a rapid wasting syndrome and die by 3 to 4 weeks of age. All of the knockout mice have a multifocal inflammatory disease in many tissues. The heart and lungs are most severely affected. Increased adhesion of leukocytes to the endothelium of pulmonary veins is the initial lesion seen at day 8 postnatally and is soon followed by perivascular cuffing as well as inflammatory infiltrates in lung parenchyma. The lesions in the heart begin as endocarditis and then progress to myocarditis and pericarditis. Within the lung, chronic inflammatory infiltrates consist of T and B lymphocytes, including plasma cells, whereas macrophages are the primary inflammatory cell type in the heart. Increased expression of major histocompatibility complex class I and II proteins is seen in pulmonary vascular endothelium as early as day 8. An immunoblastic response in mediastinal and mandibular lymph nodes and spleen is also seen. In the absence of any pathogens, this massive inflammatory disease, together with overexpression of major histocompatibility complex class I and II proteins and overproduction of immunoglobulins by lymphocytes, offers circumstantial evidence for an autoimmune etiology. (Am J Pathol 1995, 146:264–275)***

Transforming growth factor-β (TGF-β) is a key member of a superfamily of polypeptide growth and differentiation factors that actively participate in embryonic development, tissue and organ formation, cell growth and phenotype, wound healing, and immune functions.[1–3] In recent years it has become clear that TGF-β is a potent immunomodulator with profound immunosuppressive and proinflammatory actions.[4] Potent growth inhibitory actions of TGF-β on many cell types, including epithelial, endothelial, hematopoietic, and lymphoid cells, form the basis of its complex role in immunomodulation. In addition, TGF-β exerts its actions through controlling cell adhesion, extracellular matrix formation, and cytokine cross-talk and through modulating the repertoire of cell surface receptors.[1–4]

Three isoforms of TGF-β are expressed in mammals and they share a high sequence homology in the bioactive domain. However, each isoform exhibits highly conserved sequences suggesting specific individual roles for each of them. TGF-β1, a prototype of this family, generates most of the biological actions

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of the other TGF-βs. Its actions have been extensively studied *in vitro*. Recently, three different TGF-β1 "gain of function" mouse models have extended earlier findings on the regulation of cell proliferation by TGF-β1 in different cell and tissue types, and furthermore, embryological studies have demonstrated a role for the TGF-βs during development.[5–8] In order to delineate the specific roles of TGF-β1, gene knockout mouse models (loss of function) have been created.[9–11] TGF-β1 null (knockout) mice develop to term, but there is a considerable embryonic lethality. These mice remain clinically normal for the first 2 weeks after birth and then develop a fatal wasting syndrome caused by a multifocal inflammatory disease. The TGF-β1 null mice succumb to cardiopulmonary complications because of severe inflammation in these tissues. Our earlier studies indicated that increased expression of major histocompatibility complex MHC class I and II proteins in many tissues and increased adhesion of leukocytes to inner walls of veins and venules in the target tissues precede the onset of multifocal inflammation.[12,13] It has been possible to block the onset of inflammation in the mice by treating them with a combination of fibronectin peptides that interrupted increased adhesion of leukocytes *in vitro*.[13] Recently, defective interleukin 2 (IL-2) mRNA expression and IL-2 secretion in mitogen-challenged lymphoid cells *in vitro* has been reported, suggesting T cell anergy.[14]

Despite these recent findings, the mechanism of the multifocal inflammatory disease in the TGF-β1 null mice is still unclear. As a first step towards understanding the exact nature of the disease in the TGF-β1 null mice, we report here a detailed pathological analysis of the disease in many organs, establish the time course of inflammatory infiltrates, and determine the type of inflammatory cells detected in various tissues. We also show that there is an increased expression of MHC class I and II proteins in endothelial cells within pulmonary veins and venules of presymptomatic mice and a B and T cell response in the inflammatory lesions. This unique disease phenotype is compared with other known mouse models of inflammatory disorders including various autoimmune disorders.

## Materials and Methods

### Mice

The TGF-β1 gene was disrupted in embryonic stem cells derived from 129/J mice by homologous recombination and injected into recipient embryos from C57BL/6J mice as described.[9,11] Two independently targeted clones were used to generate the TGF-β1 null mice. The primary chimeras were mated with C57BL/6J mice to demonstrate the generation of heterozygous germ line chimeras. These in turn were mated together to generate homozygous TGF-β null mice. All mice were housed in a barrier facility with autoclaved food and bedding. They were free of antibodies to all common murine pathogens as described.[9]

### DNA Analysis

DNA was isolated from mouse tails by standard techniques (sodium dodecyl sulfate-proteinase K digestion). Two sets of polymerase chain reaction primers were used to diagnose the normal and the targeted allele, respectively, as described elsewhere.[15] DNA from heterozygotes reacted with both primers, but the normal and knockout mice reacted only with the normal and targeted allele, respectively.

### Pathology

Mice were sacrificed (from 16 days of gestation to 40 days of age) and a necropsy was performed. Tissues were fixed in neutral buffered formalin or Bouin's fixative or frozen in OCT compound. Fixed tissues were embedded in paraffin and sectioned at 4 to 6 μmol/L.

### FACS Analysis of Hematopoietic Cells

Cell suspensions from bone marrow, spleen, lymph nodes, and thymus were made and a panel of antibodies was then used to analyze the cell populations. Aliquots of $10^6$ cells were incubated for 30 to 60 minutes at 4 C with the following directly labeled antibodies: anti-κ-fluorescein isothiocyanate (FITC; Becton Dickinson, Mountain View CA); anti-BP-1-FITC, anti-κ-FITC, anti-IgD$^a$-FITC, anti-IgD$^b$-FITC, anti-λ-FITC (PharMingen, San Diego, CA); anti-κ-FITC, anti-μ-FITC (BioSource International, Camirillo, CA); anti-CD5-FITC, anti-Thy-1.2-FITC, anti-CD3ε-FITC, anti-TCRαβ-FITC, anti-TCRγδ-FITC, anti-Mac-1-FITC (Boehringer Mannheim, Indianapolis, IN); NK1.1-PE (gift of V. Kumar, Southwestern Medical Center, Dallas, TX), FITC-anti-CD3 (2C11; PharMingen); FITC-anti-CD8 (Lyt-2; Becton Dickinson); biotin-anti-CD4 (BRL, Bethesda, MD); FITC-anti-class I MHC (28.8.6, PharMingen); and FITC-anti-Ia (Y3P).[16–19] The following reagents were used in indirect staining assays: anti-J11D, anti-Mac-1, anti-F4/80, anti-B220, anti-I-A, and anti-IL-2 receptor. Control samples for indirect

staining were incubated only with the secondary antibody, a FITC-labeled monoclonal antibody to rat immunoglobulin κ light chain (MARK-1-FITC, Biosource International).[20–25] Unstained controls were incubated in buffer only.

### *Immunohistochemistry*

Selected frozen or Bouin's fixed tissues were used to show expression of specific antigens. The following antibodies and kits were used: mouse immunoglobulins and IgG (Vectastain Mouse Elite kit without any primary antibody), mouse IgA (Cappel, Organon Teknika, Durham, NC), human κ light chains (Biogenex, San Ramon, CA), Mac-2 (American Type Culture Collection, Rockville, MD), Mouse Ia and H-2 (Boehringer Mannheim), and Vectastain ABC kits (Vector Laboratories, Burlingame CA).

## *Results*

### *Clinical Symptoms*

Only 40% of TGF-β1 null (−/−) embryos develop to term (Table 1). The TGF-β1 −/− mice appear normal and look healthy during the first 2 weeks of postnatal life. At 12 to 14 days of age, they first developed a progressive wasting syndrome, which is mainly characterized by significant reduction in body weight gain compared with controls. These mice appeared sick with unhealthy looking fur, hunchbacked, and inactive. Figure 1 shows the body weight of TGF-β1 knockout mice compared with normal littermates. There is some variability in the rate of weight loss. Many mice are severely ill and appear as runts by three weeks of age but some can survive to 4 or 5 weeks. When the healthy pups are removed from the cage, leaving the mother to care for the sick animals only, the affected animals tend to live longer, and furthermore, affected animals in small litters tend to live longer.

Table 1. *Genotypes of Pups Born to Heterozygous Parents*

| | Normal +/+ | Heterozygous +/− | Homozygous −/− |
|---|---|---|---|
| Observed* | 230 (34%) | 380 (56%) | 66 (10%) |
| Expected | 169 (25%) | 338 (50%) | 169 (25%) |

*A total of 676 pups in 62 litters were analyzed. For observed *versus* expected, $\chi^2 = 90.02$; 1 df, $P < 0.01$.

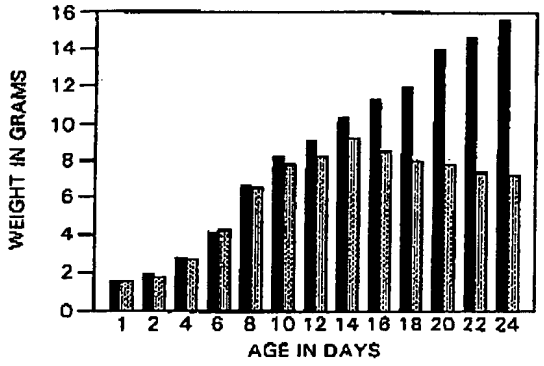



Figure 1. *Body weight of TGF-β1 knockout mice (+/+) compared with normal mice (−/−) as a function of time. The vertical axis shows body weight in grams and the horizontal axis shows the age of the animals in days. The average weight of three normal (black bars), and three knockout (dotted bars) males was compared.*

### *Tissue Distribution and Frequency of Lesions*

A total of 97 animals were examined by histopathological analysis; 45 were TGF-β1 null (−/−), 25 were TGF-β1 heterozygotes (−/+) and 27 were normal (+/+). Many tissues were examined from some mice, but in others, the review was limited to a few tissues. Lungs were examined in 34 TGF-β1 null animals, 19 heterozygotes (−/+), and 24 normal (+/+) mice. Table 2 shows the frequency of inflammatory infiltrates as detected by histopathology. The numbers in Table 2 represent the lowest possible estimate, as it is possible that a higher percentage of organs would have been scored as positive if more slides from each organ had been examined. The lungs were affected

Table 2. *Prevalence of Histopathological Lesions in TGF-β1 Null Mice*

| Tissue | 16 days postcoitum to 6 days of age | 8 to 40 days of age |
|---|---|---|
| Lung | 0/3* | 34/34 (100%) |
| Heart | 0/3 | 28/32 (87%) |
| Stomach | 0/2 | 11/14 (79%) |
| Colon | 0/2 | 10/14 (71%) |
| Pancreas | 0/3 | 14/20 (70%) |
| Liver | 0/2 | 10/14 (71%) |
| Salivary gland | 0/4 | 16/21 (76%) |
| Kidney | 0/2 | 5/20 (25%) |
| Brain | 0/2 | 3/13 (23%) |
| Eye | 0/1 | 0/3 (0%) |
| Testis | 0/3 | 0/3 (0%) |
| Thymus | 0/2 | 3/12 (25%) |
| Spleen | 0/2 | 12/18 (67%) |
| Mediastinal lymph nodes | 0/2 | 7/8 (83%) |
| Mandibular lymph nodes | 0/0 | 5/5 (100%) |

*Number of mice with lesions/number of mice examined.

in all the mice and the heart was almost always affected. Other organs that were often affected included stomach, colon, pancreas, salivary gland, spleen, and mediastinal lymph nodes. Less frequent were lesions in kidney and brain. Pathological changes in the eyes and testes were not seen. No changes were found in tissues from one TGF-β1 null fetus (16 days gestation) or from animals that were up to 6 days old (Table 2).

## *Onset and Development of Lesions in Heart and Lungs*

Lesions in the TGF-β1 knockout mice were not detected in animals that were sacrificed during the first week of life. The earliest lesions were seen at 8 days of age in lung, heart, and salivary gland. Six knockout mice were sacrificed on day 8 to determine the nature and the timing of the initial pathological changes. All six mice exhibited increased leukocyte adherence (mostly monocytes and neutrophils) to the pulmonary vein endothelium. This increased cell adhesion was most prominent from day 10 to 16 (Figure 2A), but as the inflammation progressed, fewer leukocytes adhered to the endothelial cells. Perivascular inflammation was also first found on day 8 in some mice, and phlebitis increased during the next few days with perivascular cuffs of lymphocytes, macrophages, and plasma cells (Figure 2B). The vascular wall and adventitia contained macrophages, lymphocytes, and plasma cells. As the pulmonary vascular lesions became more severe, they extended into the parenchyma as interstitial inflammation in some of the animals. In advanced lesions on or about day 21, adhesion of leukocytes was not seen (Figure 2B). The aggressiveness of the inflammatory disease varied somewhat from one animal to another. In many animals there was fulminant inflammatory disease by day 21, but less often it took 4 to 5 weeks for fulminant disease to develop. Interestingly, arteritis was not seen except in areas of pneumonia where perivascular inflammation was observed. In the lung the inflammatory infiltrates were composed of B and T lymphocytes and immunoglobulin-containing plasma cells (Figure 2B, C, and D).

RNA levels of MHC class I and II proteins have been shown to be elevated in multiple organs of symptomatic and presymptomatic TGF-β1 null mice.[12] We asked whether this overexpression could be detected by immunohistochemistry and localized to particular cells in the lungs of presymptomatic knockout mice. Increased production of MHC class I (H-2) and II (Ia) antigens was demonstrated on day 8 in pulmonary venous endothelium (Figure 2E, F) of the TGF-β1 null animals but not in similar samples from 8-day-old normal or heterozygous mice. In some of the areas where increased MHC class I and II expression was detected there were no signs of increased leukocyte adhesion. Detectable expression of the histocompatibility proteins in lung parenchyma was seen in both knockouts and controls.

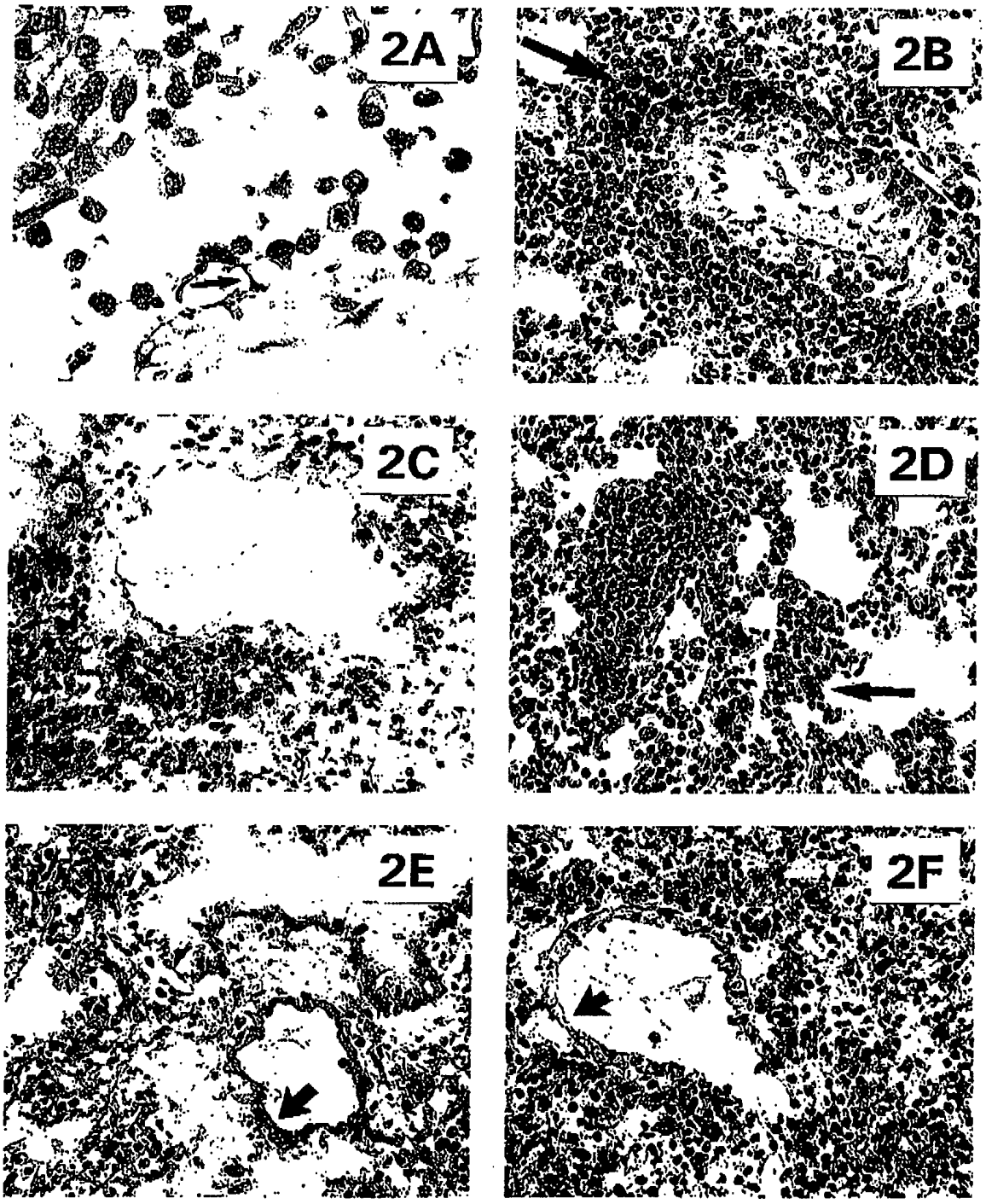
Lesions in the heart were first seen on day 8, beginning with endocardial endothelial hypertrophy and mild infiltration of mononuclear inflammatory cells (Figure 3A, B). During the next 2 weeks (animals sacrificed on days 10 to 21), the endocarditis became more severe and extended into the myocardium (Figure 3B) and pericardium (Figure 3C). Cardiac myocytes were usually normal, but some myocytes adjacent to areas of extensive inflammation contained large nucleoli and eosinophilic inclusions. The most prominent inflammatory cells in the heart lesions were macrophages and a great proportion of them were Mac-2 immunoreactive (Figure 3D). This was in sharp contrast to lesions in other organs, in particular lungs, where macrophages were much less common (Figure 2D).

## *The Lymphohematopoietic System*

The size of the lymphoid organs (thymus, lymph nodes, and spleen) was estimated by enumerating the cells in these organs. Table 3 lists the cellularity of lymphoid organs, showing a reduction in the size of the thymus in symptomatic mice (statistically significant) and increased size of axillary and inguinal lymph nodes (not statistically significant). There was no difference in spleen size when the reduction in body weight was taken into account.

The bone marrow appeared histologically normal. FACS analysis of bone marrow cells, however, showed considerable reduction in the relative number of B cells (6 to 22% are B220-positive in −/− mice *versus* 30 to 48% in +/+ and −/+ mice, n = 3), but other cell surface markers (see Materials and Methods) did not show consistent abnormalities in the ratio of the other cell lineages in the bone marrow.

Mediastinal and mandibular lymph nodes were enlarged in the knockout mice with an increased number of immunoblasts and plasma cells (Figure 4A, B). The total number of macrophages was similar in knockouts and controls by FACS analysis. Four antibodies revealed differences in proportions of lymphohematopoietic cells with defined cell surface characteristics between TGF-β1 null and normal or heterozygous animals by FACS analysis (Table 4;



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# Transforming Growth Factor-β Inhibits E-Selectin Expression on Human Endothelial Cells[1]

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ABSTRACT. Transforming growth factor-β (TGF-β), a pleiotropic cytokine that is elaborated in the active form upon co-culture of endothelial cells and pericytes or smooth muscle cells, has been shown to decrease the adhesiveness of endothelial cells for neutrophils, lymphocytes, and tumor cells. The mechanism whereby TGF-β inhibits the adhesiveness of human endothelial cells was investigated. TGF-β inhibited the basal E-selectin (formerly ELAM-1) expression by 55 ± 7% and TNF-stimulated expression by 57 ± 4%. Similar decreases of IL-1-stimulated expression were also seen. Peak inhibition was seen at TGF-β doses between 0.2 and 2 ng/ml. Both TGF-$\beta_1$ and -$\beta_2$ were functional. The effectiveness of TGF-β in inhibiting E-selectin expression was dependent on cell density and incubation time. TGF-β also inhibited E-selectin mRNA levels in endothelial cells. TGF-β had no effect on the expression of VCAM-1 and ICAM-1, but was additive with IL-4 in inhibiting the expression of E-selectin. The expression of E-selectin has been shown to mediate several aspects of the inflammatory response involving neutrophils and memory T lymphocytes. Perivascular TGF-β appears to act as an inhibitor of the expression of the endothelium-specific selectin, E-selectin, and therefore of inflammatory responses involving neutrophils and (a subset of) lymphocytes. *Journal of Immunology,* 1993, 150: 4494.

The adhesion of blood cells to endothelium is an essential process during the development of the inflammatory response. The treatment of EC[3] with TNF-α (1) or IL-1 (2) increases the adhesion of WBC due to the de novo or increased expression of adhesion molecules. In the case of neutrophils, the increased adhesion to IL-1- or TNF-treated HUVEC is due to the expression on HUVEC of the adhesion molecule, E-selectin, previously known as ELAM-1 (3–5). E-selectin is only expressed in EC and, in keeping with the function of all the three members of the selectin family, may also be responsible for the capture of neutrophils from the rapidly flowing axial blood stream to the marginated or rolling pool of cells (6). E-selectin is thought to provide the shear-resistant form of adhesion that is necessary before the integrin-mediated steps of adhesion and transmigration can begin to operate (7). E-selectin also stimulates the recognition by neutrophils of C-coated particles and may act as a chemotactic stimulus for neutrophils (8). In vivo E-selectin expression is seen at sites of inflammation mainly in the skin (9). It is also expressed in various tissues after inflammatory assaults and in the vasculature of lymphoid tumors (10, 11).

E-selectin also supports the adhesion of memory and skin homing T lymphocytes (12, 13), but not other types of lymphoid cells. Lymphocyte adhesion is supported by two other adhesion molecules, ICAM-1 and VCAM-1, the expression of which is also induced by TNF and IL-1 (14, 15). Whereas ICAM-1 is constitutively expressed, VCAM-1 is either absent or present in very low levels in resting EC (15). VCAM-1 expression, however, has also been observed in vivo at sites of chronic inflammation (16, 17),

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[3] Abbreviations used in this paper: EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; TIE, TGF-β inhibitory element; nt, nucleotide; ECGS, endothelial cell growth supplement; TGF-β, transforming growth factor-β.

notably in the aorta at sites of atheroma formation (17), where presumably it mediates the influx of T lymphocytes and monocytes into these lesions. The regulation of VCAM-1 expression has been shown to differ from that of E-selectin. IL-4 induces VCAM-1 expression, whereas it inhibits TNF or IL-1 induction of E-selectin (14, 18).

TGF-β and related cytokines have been shown to be involved in processes of tissue formation and also to have immunosuppressive properties (19, 20). Active TGF-β is made by EC in vitro when they are co-cultured with pericytes or smooth muscle cells (21, 22). Inasmuch as these cells are in juxtaposition in vivo, it is suggested that the TGF-β found perivascularly is in the active form. Vascular TGF-β has been hypothesized to control the composition of the extracellular matrix deposited by EC, pericytes or smooth muscle cells, and fibroblasts. We have previously shown that treatment of HUVEC with TGF-β decreases basal adhesiveness to neutrophils and lymphocytes (23, 24). In addition, TGF-β inhibited neutrophil and lymphocyte adhesion stimulated by the cytokines TNF and IL-1β (23, 24) and supported the concept that perivascular TGF-β has an anti-inflammatory role. The inhibitory effects of TGF-β on adhesion were evident on recently explanted (or "young") HUVEC but were absent or much weaker on cells that had been in culture for more than approximately 2 wk ("old"). Subsequently, TGF-β has been shown to inhibit the adhesion of murine lymphocytes to Peyers patch endothelium and high endothelial venule cells (25) and of murine tumor cells to IL-1- or TNF-treated murine EC (26). These results suggested that TGF-β is an important regulator of endothelial function. We now show that TGF-β inhibits the expression of the endothelium-specific selectin, E-selectin, potentially providing an important mechanism for the prevention of inflammation.

## Materials and Methods

### EC

EC were extracted from umbilical veins by collagenase treatment according to a modified version of Wall et al. (27). Cells were grown in 25-$cm^2$ gelatin-coated Costar flasks (Cambridge, MA) in endotoxin-free M199 (Cytosystems, Sydney, Australia), 20% FCS (PA Biologicals, Sydney, Australia), 20 mM HEPES, with sodium pyruvate, nonessential amino acids and Fungizone. Young cells were used 2 to 5 days after establishment of culture. For additional growth, cells were harvested by trypsin-EDTA treatment (Flow, Australia) replated into 75-$cm^2$ gelatin-coated flasks in endotoxin-free M199, 20% FCS, 20 mM HEPES, sodium pyruvate, nonessential amino acids, and Fungizone with the addition of ECGS (Collaborative Research, Bedford, MA) and heparin (Sigma, St. Louis, MO), both at a final concentration of 50 μg/ml. Microvessel EC were prepared from neonatal foreskins according to the method of Marks et al. (28). Cells were frozen in liquid nitrogen at 1 to 2 × $10^6$/vial at passages 2 to 6 and thawed as required. Medium for growth and maintenance of these cells was M199 with Earle's salts, 25 mM HEPES, 50% human serum, sodium bicarbonate, 2 mM glutanine, Fungizone, penicillin, streptomycin, 3.3 × $10^{-4}$ M cAMP, ECGS (50 μg/ml), and heparin (50 μg/ml).

### Cell surface Ag expression

EC were plated onto gelatin-coated 24-well tissue culture trays (Nunc, Denmark) at $10^5$ cells/well in 500 μl of HUVEC medium without ECGS or heparin. TGF-β was added at the time of plating. In some cases, TNF or IL-1β was added 19 to 20 h later for 4 to 5 h, giving a total incubation time of approximately 24 h. After this, medium was removed and the cells washed twice with fresh medium, and 200 μl of antibody directed to E-selectin, VCAM-1, or ICAM-1 were added. Cells were incubated for 30 min at 37°C and washed twice, and then sheep anti-IgG $Fab_2$-FITC-labeled antibody (Silenus, Victoria, Australia) added in a volume of 200 μl. The cells were incubated on ice for 30 min, washed three times in PBS, harvested by trypsin-EDTA treatment, pelleted, and resuspended in fixative (1% formaldehyde, 2% glucose, 5 mM sodium azide in PBS, pH 7.3). The fluorescence profiles were analyzed by flow cytometry using an EPICS Profile II; 10,000 cells/group were analyzed. The above method for fluorescence staining of attached cells was tested in parallel with cells detached before labeling. We found no difference in the level of expression of E-selectin, VCAM-1, or ICAM-1 with these two methods. Since fewer cells could be used to stain attached cells, this was chosen for all experiments described here.

### ELISA assay for E-selectin expression

Freshly trypsinized EC were plated onto collagen-coated 96-well flat bottomed trays at 2 × $10^4$ cells/well and incubated. TGF-β was added to wells at plating and TNF-α the next day 4 h before assay. For assay, medium was flicked off the plates and anti-E-selectin antibody incubated at 37°C for 1 h. Supernatants were flicked and plates washed once with RPMI + 10% FCS. The mouse Ig signal was amplified with rabbit anti-mouse Ig (Dako 2412) at 37°C for 30 min followed by one wash with assay medium and detected with goat anti-rabbit horseradish peroxidase (Dako P448) at 37°C for 30 min, followed by three washes. Positive signals were detected with *O*-phenylene diameric (Sigma P-1526) 1 mg/ml in 0.1 M citrate, pH 6.5, + 0.03% $H_2O_2$, at room temperature for 8 min and quenched with 1 M $H_2SO_4$, and OD read at 490 nm.

### RNase protection assays

A 348-bp fragment of E-selectin cDNA (a gift from Dr. B Seed, Boston, MA) spanning nt 1–348 (5), was subcloned into the vector pGEM-1. The plasmid was linearized 74 bp 3′ of the cloning site and transcribed using SP6 RNA polymerase in the presence of [$^{32}$P]UTP as previously described (29) to generate a full length cRNA of 422 bases. A human β-actin cRNA probe of 330 bases containing 120 complementary nt to the human β-actin mRNA was used as an internal standard. The human β-actin cRNA probe used had approximately two times higher specific radioactivity than the E-selectin cRNA probe.

Total RNA from EC was isolated according to the method of Chomczynski and Sacchi (30); 5 μg of total RNA were hybridized to 20,000 cpm of each of the E-selectin and β-actin cRNA probes followed by digestion with 40 μg/ml RNase A essentially as described (31). The protected fragments of 348 bases (E-selectin) and 120 bases (β-actin) were resolved by electrophoresis on 6% polyacrylamide gels containing 8 M urea.

The amount of radioactivity in each band was visualized and quantitated using a Molecular Dynamics Phosphorimager. The extent of stimulation or inhibition of E-selectin mRNA by TNF or TGF-β was calculated after normalization using the β-actin mRNA as an internal control.

### Antibodies

The antibody 1.2B6 (IgG1) (32), which reacts with E-selectin, was kindly provided by Dr. Dorian Haskard, Hammersmith Hospital, London, United Kingdom. The anti-VCAM-1 antibody was kindly supplied by Dr. Mike Gallatin and Dr. Boris Masinovsky, ICOS, Seattle, WA. The anti-TGF-β antibody (lot 8755.81) was supplied by Genentech, South San Francisco, CA; 1 mg of the antibody neutralizes 160 ng TGF-$\beta_1$, TGF-$\beta_2$, and TGF-$\beta_3$.

### Cytokines

TNF-α (lot S9010AX; sp. act., $6.27 \times 10^7$ U/mg) and TGF-β (lots 8987–53 and G098AD) were kindly supplied by Genentech. Porcine TGF-$\beta_1$ and -$\beta_2$ were obtained from R&D Systems (British Biotechnology Ltd., Oxford, United Kingdom). IL-1β ($10^8$ thymocyte mitogenesis U/mg) was kindly supplied by Immunex, Seattle, WA. All reagents contained less than 3 Ehrlich U/mg of LPS as detected by limulus amoebocyte assay.

### Statistics

Significance was determined by the unpaired two-tailed *t*-test.

Table I
*Effect of TGF on basal and stimulated E-selectin expression*

| TGF-β (ng/ml) | E-Selectin | | | |
|---|---|---|---|---|
| | 0 | 0.2 | *p* value | % Inhibition |
| Basal ($n$ = 11) | 1.7 ± 0.6[a] | 0.9 ± 0.4 | 0.008[b] | 55 ± 7[c] |
| TNF stimulated ($n$ = 16)[d] | 20.0 ± 4.3 | 7.1 ± 1.6 | 0.002 | 57 ± 4 |

[a] Mean fluorescence expressed as mean channel number ± SEM of 10,000 cells.
[b] $p$ value of difference between 0 and 0.2 ng/ml TGF-β.
[c] Inhibition was calculated for each experiment and mean then calculated.
[d] These 16 experiments include all 11 used for "basal" studies. The results were not significantly different if the additional five experiments for which there were no corresponding basal studies were excluded.

## Results

### Effect of TGF-β on basal expression of E-selectin

EC derived from umbilical veins have a low expression of E-selectin for about the first week in culture, perhaps due to cytokines secreted by passenger leukocytes (J. R. Gamble and M. A. Vadas, unpublished observations). We noted that this expression declines with passage in culture as the number of contaminating cells also declines. In young EC as measured by flow cytometry, 23 ± 8% (mean ± SEM $n$ = 6) of the cells were positive for E-selectin, with a mean intensity of positive cells of 5.7 ± 0.2 channels. In old EC, only 2 ± 1% ($n$ = 4) of cells were positive, with a mean intensity of positive cells of 2.8 ± 0.7 channels. TGF-β was added to young HUVEC for 24 h, and the expression of E-selectin was determined by flow cytometry. TGF-β inhibited basal mean E-selectin expression by 55 ± 7% (mean ± SEM of 11 separate experiments) (Table I). Figure 1*A* gives an example of the flow cytometry profile of young HUVEC cultured in the presence or absence of 0.2 ng/ml TGF-β. The inhibition was maximal with 0.2 ng/ml and was less at higher and lower doses (Fig. 2*A*). The bell shaped dose-response curve was a consistent observation, but the most effective dose of TGF-β varied between 0.2 and 2.0 ng/ml, depending on the batch of TGF-β used. A bell-shaped dose-response curve to TGF-β is also seen with neutrophil or lymphocyte adhesion (23, 24) in the inhibition of smooth muscle cell proliferation (33), and in the TGF-$\beta_1$-induced neutrophil chemotaxis (34).

### Effect of TGF-β on TNF-stimulated expression of E-selectin

TNF stimulates the expression of E-selectin on EC, with peak expression being seen 4 to 6 h after the addition of TNF (4, 5). In young cells, after 1 U/ml TNF, 92 ± 9% ($n$ = 6) of cells became positive with a mean intensity of 17.7 ± 2.4 channels. In old cells, only 49 ± 4% of cells became positive with a mean intensity of the positive population of 5 ± 1 channels. TGF-β added 20 h before TNF inhibited this effect of TNF on young cells, and as with

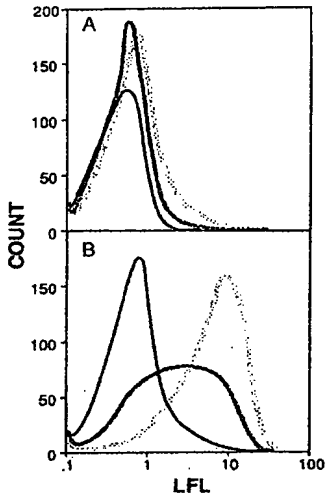


**FIGURE 1.** Flow cytometry profiles of the expression of E-selectin (*light lines*) in young (3 days in culture) HUVEC that were not (*A*) or were (*B*) treated for 4 h with 1 U/ml TNF. The effect of 0.2 ng/ml TGF-β added 24 h before assay is also shown (*dark lines*). *Black lines* show fluorescence with control antibody. *Vertical axis* shows cell numbers in each channel and *horizontal axis*, the intensity of fluorescence on a 3-decade scale.

basal E-selectin expression, 0.2 ng/ml TGF-β gave maximal inhibition (Fig. 2*B*). Inhibition was seen at various doses of TNF (Fig. 3), however the largest inhibitions were observed at submaximal doses of TNF. In a series of 16 experiments, 0.2 ng/ml TGF-β inhibited 1 U/ml TNF-stimulated E-selectin expression by a mean of 57 ± 4% (Table I). The flow cytometry profile (Fig. 1*B*) of a typical experiment shows the shift in E-selectin expression after TNF treatment and its inhibition by TGF-β. In all experiments, flow cytometry profiles showed a single bell-shaped curve of E-selectin expression after TNF induction. Therefore, in all other experiments, fluorescence levels are reported as the mean channel fluorescence. Similar levels of increased E-selectin expression with TNF treatment and decreased E-selectin expression after TGF-β were observed in an ELISA in which no trypsinization of the cells was requaired (data not shown). As previously shown for adhesion (24), TGF-β was effective in inhibiting E-selectin expression when added 24 h, 15 h, but not 6 h or 3 h before assay (data not shown). The effect of TGF-β was not evident if added 48 h before assay (Table II, group II compared to group III), however, this was most likely due to either exhaustion of the amount or effectiveness of TGF-β present or conversion to an inactive (35) form, since re-addition of TGF-β after 24 h of culture resulted in equivalent levels of inhibition of E-selectin as that seen with only 24-h incubation (Table II, groups IV and V). TGF-β inhibited IL-1-induced E-selectin expression in a similar fashion to that of TNF (data not shown). A mAb against TGF-β prevented the inhibition of E-selectin expression (Table III), and recombinant human and purified porcine TGF-$\beta_1$ were both active, as was purified porcine TGF-$\beta_2$ (Table IV). The ability of TGF-β to inhibit TNF-induced E-selectin expression was dependent on the density of the cells. Cells plated at high density (Table V) or allowed to grow to high density before the addition of TGF-β (data not shown) were less responsive than cells plated at lower cell numbers. For three experiments, the mean inhibition for cells plated at $1 \times 10^5$/well was 64.1 ± 3.5 and for cells at $2 \times 10^5$/well was 20.1 ± 4.5 ($p = 0.009$). The effect of TGF-β was also seen in old EC, however the decrease observed was of a lesser magnitude, was not observed in all EC lines tested and, if seen, was observed only at a single concentration of 0.2 ng/ml of TGF-β (Table VI). For 13 experiments with old cells, 0.2 ng/ml of TGF-β-inhibited TNF-α stimulated E-selectin expression by 22.6 ± 6.3%. Cell lines of capillary endothelium derived from human foreskin were also tested for E-selectin regulation by TGF-β. Of four lines analyzed, two showed no change in E-selectin and two showed a decrease (by 98 and 62%), with 0.2 ng/ml of TGF-β in TNF-induced E-selectin expression.

## TGF-β inhibits E-selectin mRNA accumulation

E-selectin mRNA was measured by RNase protection in young or old EC that were or were not pretreated with 0.2 ng/ml TGF-β (Fig. 4). The higher basal and stimulated expression of E-selectin mRNA in young rather than old EC is evident, as is the inhibitory effect of TGF-β. Using a phosphorimager to quantitate the amount of radioactivity, the mean E-selectin mRNA levels in three experiments (calculated as a ratio of E-selectin to actin) was obtained (Table VII). A 7.1 ± 1.5 (mean ± SEM, $n = 3$, $p = 0.008$)-fold higher basal expression of E-selectin mRNA was observed in young EC compared to old EC. TNF (1 U/ml) stimulated expression by 6.7 ± 0.9-fold in young and 17.4 ± 0.8-fold in old cells. Notably the level of E-selectin mRNA and surface expression after any dose of TNF was always higher in young than old EC (data not shown). However, inasmuch as young EC also always had higher basal expression, correspondingly the increase was about twofold less in young than old HUVEC. TGF-β inhibited basal E-selectin mRNA in young cells in every experiment and by a mean of 36 ± 10% ($p = 0.03$, $n = 3$), but not significantly ($p = 0.3$) in old ($n = 6$) cells. TNF-induced E-selectin mRNA was inhibited by TGF-β in each experiment with young EC (mean inhibition, 23 ± 6%, $n = 3$, $p = 0.01$) but was more variable in old cells (mean inhibition, 25 ± 9%, $n = 6$, $p = 0.06$).

## Effect of TGF-β on VCAM-1 expression

We have previously shown that TGF-β did not alter the expression of ICAM-1 or VCAM-1 on HUVEC (24). We

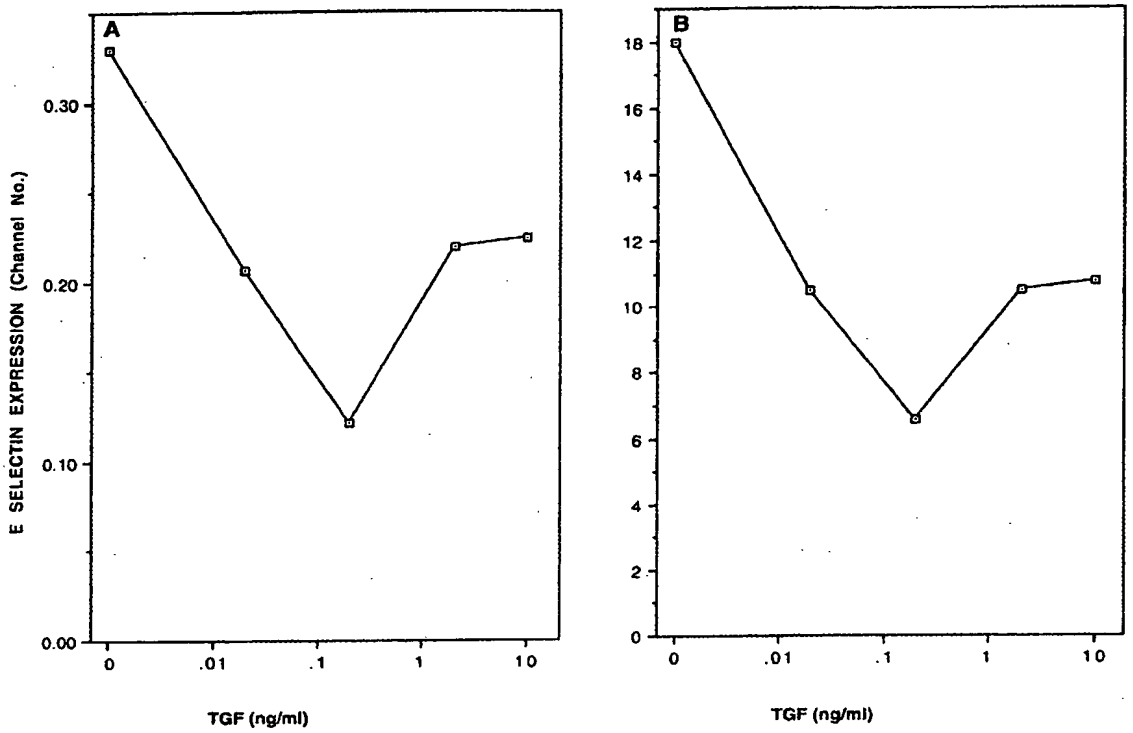


**FIGURE 2.** Effect of a range of doses of TGF-β on mean E-selectin expression by young EC. *A*, Basal expression; *B*, expression stimulated by 1 U/ml TNF. Ten other experiments gave similar results, although on occasion peak inhibition was seen with 2 ng/ml TGF-β.

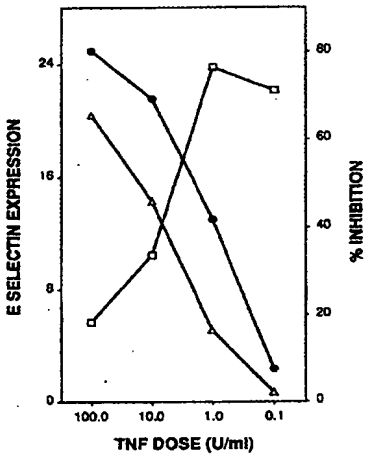


**FIGURE 3.** Effect of TGF-β on E-selectin expression stimulated by various doses of TNF. Expression (mean channel number) in the absence of TGF-β (●) or with 0.2 ng/ml TGF-β (△) is shown. The calculated percentage inhibition (□) is also shown. The experiment was repeated with similar results.

now confirm the findings on VCAM-1, in which case TGF-β did not significantly decrease basal expression (without TGF-β mean expression was 6.10 ± 2.19, and with TGF-β 5.89 ± 1.73, $n = 5$) nor TNF-induced expression (without TGF-β mean expression was 16.70 ± 5.27 and with TGF-β 15.67 ± 5.06, $n = 8$) in young EC. In the latter instance, we did however note a significant decrease in two of the eight lines examined.

Table II
*Effect of time of addition of TGF-β on E-selectin expression*[a]

| Group | Time of Addition of TGF-β[b] | TNF[c] (U/ml) | E-Selectin Expression after: 24-h culture | 48-h culture |
|---|---|---|---|---|
| I | Nil | 0 | 1.05[d] | 0.67 |
| II | Nil | 1 | 9.73 | 18.80 |
| III | At plating | 1 | 3.33 (66)[e] | 17.82 (5.2) |
| IV | 24 h after plating | 1 | | 7.04 (63) |
| V | At plating and 24 h after plating | 1 | | 8.39 (55) |

[a] Data are representative of three similar experiments.
[b] TGF-β used at 0.2 ng/ml.
[c] TNF added 4 h before assay.
[d] Mean fluorescence expressed as mean channel number of 10,000 cells.
[e] Numbers in parentheses, percentage inhibition compared to no TGF-β.

## TGF-β and IL-4 show additive effects at decreasing E-selectin expression

It was previously shown (14) that IL-4 added 20 h before TNF inhibits the induction of E-selectin. We now confirm these findings and in addition observe a decrease in basal

Table III
*Inhibition of TGF-β by anti-TGF-β antibodies*

| TGF-β[a] (ng/ml) | Antibody[b] (μg/ml) | TNF[c] U/ml) | E-Selectin Experiment 1 | E-Selectin Experiment 2 |
|---|---|---|---|---|
| 0 | 0 | 0 | 0.4[d] | 1.6 |
| 0 | 0 | 1 | 7.6 | 11.0 |
| 0.2 | 0 | 1 | 4.4 | 8.3 |
| 0.2 | 56 | 1 | 9.1 | 12.1 |

[a] TGF-β was added 24 h before assay.
[b] Anti-TGF-β was added to TGF-β for 30 min at room temperature before the mixture was added to HUVEC.
[c] TNF was added 4 h before assay.
[d] Mean fluorescence expressed as mean channel number of 10,000 cells.

Table IV
*Comparison of the effect of TGF-$\beta_1$ and $\beta_2$ on TNF-stimulated E-selectin expression*

| TGF-β Type | TGF-β ng/ml | TNF (U/ml) | E-Selectin Experiment I | E-Selectin Experiment II |
|---|---|---|---|---|
| — | 0 | 0 | 1.2[a] | 0.7 |
| — | 0 | 1 | 11.9 | 6.8 |
| rh$\beta_1$[b] | 2 | 1 | 4.9 | 2.3 |
| rh$\beta_1$[b] | 0.2 | 1 | 7.1 | 4.0 |
| p$\beta_1$[c] | 2 | 1 | 5.7 | 2.8 |
| p$\beta_1$[c] | 0.2 | 1 | 5.8 | 3.7 |
| p$\beta_2$[c] | 2 | 1 | 6.2 | 3.9 |
| p$\beta_2$[c] | 0.2 | 1 | 8.4 | 5.3 |

[a] Mean fluorescence expressed as mean channel number of 10,000 cells.
[b] rh$\beta_1$, Recombinant human TGF-$\beta_1$. For this batch of human rTGF-β, maximum inhibition was generally seen at 2.0 ng/ml.
[c] p$\beta_1$ or p$\beta_2$, Purified porcine TGF-$\beta_1$ or -$\beta_2$

Table V
*Effect of cell density on EC responsiveness to TGF-β[a]*

| No. EC Plated/Well | TGF-β (ng/ml) | TNF-α[b] (U/ml) | E-Selectin Expression after 24-h Exposure to TGF-β |
|---|---|---|---|
| 1 × 10$^5$[c] | 0 | 0 | 0.53[d] |
| | 0 | 1 | 26.68 |
| | 0.2 | 1 | 9.85 (63)[e] |
| 2 × 10$^5$[c] | 0 | 0 | 0.22 |
| | 0 | 1 | 17.05 |
| | 0.2 | 1 | 12.36 (28) |

[a] Data are representative of three similar experiments.
[b] TNF-α added 4 h before assay.
[c] EC plated at 1 × 10$^5$/well formed a monolayer but did not assume the cobblestone morphology after 24 h of culture. When plated at 2 × 10$^5$ cells/well, EC showed a cobblestone confluent morphology after 24 h of culture.
[d] Mean fluorescence expressed as the mean channel number of 10,000 cells.
[e] Numbers in parentheses, percent inhibition compared to no TGF-β.

E-selectin expression in the presence of IL-4. We also note that the magnitude of the decrease of E-selectin expression is greater with IL-4 alone than with TGF-β alone, but show that at maximal doses of IL-4, TGF-β has an additional suppressive effect on E-selectin expression (Table VIII).

## Discussion

The expression of adhesion proteins by EC is essential in the development of inflammatory responses. We now show

Table VI
*Effect of TGF-β on E-selectin expression in young and old HUVEC[a]*

| HUVEC | TNF (U/ml) | 0 TGF-β | 0.2 ng/ml TGF-β | % Inhibition[b] |
|---|---|---|---|---|
| Young | 0 | 0.2[c] | 0.07 | 70 |
| Young | 1 | 5.2 | 2.4 | 53 |
| Old | 0 | 0 | 0.04 | 0 |
| Old | 1 | 0.8 | 0.6 | 22[d] |

[a] Data are representative of 10 to 13 similar experiments.
[b] The calculation for percent inhibition included the second decimal point where necessary.
[c] Mean fluorescence expressed as mean channel number of 10,000 cells.
[d] This inhibition was only observed at 0.2 ng/ml, whereas in young HUVEC, inhibition was seen with doses between 0.02 and 10 ng/ml (see Fig. 2).

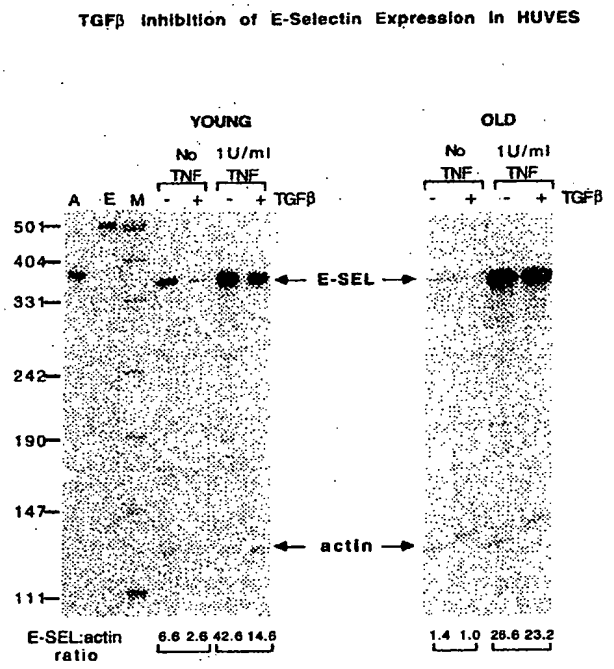


**FIGURE 4.** RNase protection assay of E-selectin and actin mRNA expression in young EC. The basal expression of E-selectin, its induction by 1 U/ml TNF, and the inhibitory effect of 0.2 ng/ml TGF-β are demonstrated; 5 μg of total RNA from EC were hybridized to $^{32}$P-labeled E-selectin and β-actin cRNA probes followed by RNase A digestion, PAGE, and autoradiography. The autoradiogram shows full length protected fragments by E-selectin and β-actin mRNA (indicated by *arrowheads*) as well as three minor fragments between m.w. markers of 147 bases and 190 bases. These three minor bands are probably the result of polymorphism within the E-selectin mRNA. *Lane A*, Full length actin cRNA probe; *Lane E*, full length E-selectin cRNA probe; *Lane M*, m.w. markers. The E-selectin:actin ratio was obtained by quantitating the E-selectin and action bands using a phosphorimager.

that TGF-β inhibits the expression of E-selectin on venous (HUVEC) EC. The inhibition is seen both with basal expression of E-selectin, seen in recently explanted or young HUVEC, and of TNF- and IL-1-stimulated expression in

Table VII
*Inhibition of E-selectin mRNA by TGF-β in young and old HUVEC*

| TGF-β (ng/ml) | TNF (U/ml) | E-Selectin:Actin Ratio | |
|---|---|---|---|
| | | Young | Old |
| 0 | 0 | 7.4[a] | 1.1 |
| 0 | 1 | 50.0 | 19.7 |
| 0.2 | 0 | 4.5 | 0.7 |
| 0.2 | 1 | 38.0 | 13.1 |

[a] The amount of radioactivity was quantitated using a phosphorimager. Arithmetic mean of three experiments.

Table VIII
*Additive effect of TGF-β and IL-4 on E-selectin expression*[a]

| TGF-β (ng/ml) | IL-4 (ng/ml) | TNF (U/ml) | E-Selectin | |
|---|---|---|---|---|
| | | | Experiment I[b] | Experiment II[b] |
| — | — | — | 6.7[c] | 4.0 |
| 0.2 | — | — | 4.2 | 1.9 |
| — | 10 | — | 2.4 | 1.3 |
| 0.2 | 10 | — | 1.8 | 1.0 |
| — | — | 1 | 13.1 | 11.4 |
| 0.2 | — | 1 | 8.5 | 4.5 |
| — | 10 | 1 | 5.7 | 8.8 |
| 0.2 | 10 | 1 | 4.3 | 2.5 |

[a] Data are representative of four similar experiments.
[b] Young HUVEC were used for these experiments.
[c] Mean fluorescence expressed as mean channel number of 10,000 cells.

both young and old EC and in two of four capillary EC lines tested. The extent of inhibition was approximately the same as the inhibition of adhesion that we had observed previously (23, 24). The presence of immunoreactive TGF-β around blood vessels in vivo (36) and the production of active TGF-β by a co-culture in vitro of the cellular components of blood vessels, EC and pericytes (21), or smooth muscle cells (22) suggests that this is an important mechanism that tonically inhibits E-selectin expression and therefore the types of inflammation in which E-selectin is involved.

TGF-β inhibits steady state E-selectin mRNA, suggesting that TGF-β may exert its effect at the transcriptional level or at the level of mRNA stability. The necessity for TGF-β to be present at least 10 h before the addition of TNF or IL-1 argues for synthesis of a factor that prevents transcription. This is in accordance with the observations that cyclophosphamide abolishes the inhibitory effect of TGF-β on adhesion in a murine system (26). Bereta et al. (26) also showed that okadaic acid, an inhibitor of phosphatases, reverses the effect of TGF-β on adhesion in a murine system, suggesting that dephosphorylation of a protein is a step in this pathway.

The transcription rates of several genes are inhibited by TGF-β. Based on sequence homology in the promoters of these genes, Kerr et al. (37) have suggested a putative TIE with the consensus sequence, GNNTTGGtGa (Table IX).

Table IX
*TGF-β inhibitory element-like sequences in the E-selectin promoter*

| nt[a] | TIE-Like Sequence in E-Selectin Promoter |
|---|---|
| Consensus | GNNTTGGtGa[b] |
| −74 to −65 | GATGTGGACA |
| −98 to −89 | CCATTGGGGA |
| −160 to −151 | GAGTTTCTGA |
| −194 to −185 | GGCATGGACA |
| −460 to −451 | GAATTGGCAG |
| −800 to −791 | GAGATGGCGT |
| −824 to −833 | GGCATGGTGG[c] |

[a] The nt numbering used is according to the method of Whelan et al. (38).
[b] *n* = any nt; capital letters = invariant nt; lower case letters = preferred nt.
[c] This sequence lies on the opposite strand.

They have also demonstrated the presence of a TGF-β-inducible nuclear protein complex that specifically binds to the TIE in the transin/stromelysin promoter. We have compared the E-selectin promoter sequence (38) (European Molecular Biology Laboratories database) from nt −900 to the start site of transcription (+1) with the TIE consensus sequence. Seven TIE-like elements are present in the E-selectin promoter (Table VIII). Whether any of these sequences is sufficient for binding of the nuclear protein complex that interacts with the TIE remains to be determined. Interestingly, two of these sequences (nt −74 to −65 and nt −98 to −89) overlap with the CAAT-box and the NF-κB consensus sequence suggested to be responsible for the TNF-induced transcription of E-selectin (39) and, hence, TGF-β may inhibit E-selectin transcription by inducing a nuclear protein complex that competes with the CAAT-binding protein and/or NF-κB for their respective binding sites.

The other striking observation in this paper is the constitutive expression of E-selectin as seen in young EC (Tables IV and V). This could be due to TNF or IL-1 made by passenger leukocytes. However, it has been well described that TNF, IL-1, and endotoxin induce E-selectin expression that peaks at 4 to 6 h and largely disappears by 24 h (40). The expression of E-selectin in young EC is not transient but maintained for about 1 wk, suggesting that a factor other than TNF, IL-1, or LPS is involved. It should be noted that the expression of E-selectin in skin EC is present in chronic skin diseases, such as atopic dermatitis (10). One possibility is that IFN-γ, which by itself has no effect on E-selectin expression, but, in conjunction with TNF can maintain or stabilize E-selectin expression (41), is responsible. However, IFN-γ only increases the percent of EC showing positivity and has no effect on the degree of positivity of the cells (41). In the case of young EC, both the percent positivity and the amount of E-selectin-positive cells are increased compared to old EC. Furthermore, these young EC are capable of expressing more E-selectin mRNA than old EC regardless of the dose of TNF used, suggesting that a

factor other than IFN-γ is responsible. Preliminary experiments from our laboratory have shown that supernatant from young EC can stabilize and increase the expression of E-selectin on multi-passaged EC (J. R. Gamble and M. A. Vadas, unpublished observations). Regardless of the nature of this factor, we note that TGF-β inhibits or eliminates basal E-selectin expression and mRNA in young, but not in old, EC. The reason for the loss of responsiveness to TGF-β with regard to regulation of adhesion and E-selectin expression is not known at present. One possibility is that old EC may actually be secreting active TGF-β. However, supernatant taken from confluent old EC does not result in inhibition of TNF-induced E-selectin expression in young cells (data not shown).

The effect of TGF-β on E-selectin expression is dependent on cell density. E-selectin expression is significantly inhibited by TGF-β in EC that are semiconfluent. Cells that are either plated down at high cell density or that are allowed to grow to high density before the addition of TGF-β respond poorly. This density dependence of the activity of TGF-β has been reported for fibroblasts (42, 43), smooth muscle cells (44), and EC (35). The mechanism for the variation in response to TGF-β is not known but may depend on the extracellular matrix deposited by the cells, the proliferative potential of EC, or the expression of TGF-β binding proteins.

Although TGF-β has been shown to alter the expression of many proteins, notably those of the extracellular matrix and of enzymes responsible for degrading these (19, 20), this is the first description of the effect of TGF-β on selectins. The effect of TGF-β on adhesion proteins is selective, inasmuch as there is no significant change in the expression of the Ig-like adhesion protein, ICAM-1 or VCAM-1 (23). The lack of effect on VCAM-1 is notable, inasmuch as it is another adhesion molecule with an absent or low basal expression in which expression is induced by TNF or IL-1.

The selectivity of TGF-β for E-selectin expression vs ICAM-1 and VCAM-1 may be important given the different roles for these EC adhesion molecules. E-selectin binds neutrophils, eosinophils, monocytes, and a subset of T lymphocytes, and is believed to play an essential role in the capture of leukocytes from the circulation. ICAM-1 and VCAM-1 molecules bind a similar spectrum of cells (although VCAM-1 does not bind neutrophils (18)) and interact with the $\beta_2$ and $\alpha_4\beta_1$ integrins, respectively, allowing the adhesion of cells at low shear forces and their transmigration from blood to tissue. Indeed we have noted that TGF-β does not inhibit the capacity of HUVEC to allow cellular transmigration (W. B. Smith, J. R. Gamble, and M. A. Vadas, unpublished observations). Thus, it is envisaged that in areas where blood stagnates or shear forces are low, the inhibitory effect of TGF-β will not be significant, and that the main site of action of TGF-β is likely to be in capillaries and postcapillary venules. The dominant role of E-selectin in skin (9, 10) and in the adhesion of skin homing and memory T lymphocytes (12, 13) suggests that the powerful in vivo anti-inflammatory and immunosuppressive action of TGF-β (45) is mediated in part by inhibiting the localization of T cells to the site of antigenic challenge and hence, of the orchestration of a specific immune response.

IL-4 is similar to TGF-β in that it inhibits the expression of E-selectin, however, it differs from TGF-β in being a strong inducer of the expression of VCAM-1 (14). Indeed, IL-4 and TGF-β are to some extent additive in inhibiting E-selectin expression (Table VIII), suggesting that their combined presence could eliminate the contribution of E-selectin to inflammatory responses. The failure to find E-selectin in certain tissues such as kidney (M. A. Vadas and D. Gillis, unpublished observations) may be due to the combined effect of these cytokines.

TGF-β is a cytokine that is made by blood vessel cells, the expression of which increases after arterial injury (36). The therapeutic administration of TGF-β has been shown to diminish the size of experimental myocardial infarction (46), suggesting an important role for perivascular TGF-β. The importance of TGF-β in the vasculature is further substantiated by the results with TGF-$\beta_1$-deficient mice in which multifocal inflammatory disease with gross cellular infiltration is seen (47). Our results showing that TGF-β inhibits the expression of E-selectin on EC suggest that TGF-β may be an important molecule in the control of EC function.

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# Increased expression of transforming growth factor-β in renal disease

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Transforming growth factor-β (TGF-β) is a multifunctional cytokine and a major regulator of tissue repair and extracellular matrix. Recent studies show that TGF-β overexpression in experimental and human kidney diseases leads to progressive glomerular and tubulointerstitial scarring and renal failure. New evidence suggests that angiotensin-converting enzyme inhibitors and a low-protein diet may slow the progression of chronic kidney diseases in part by suppressing TGF-β overexpression. New therapies may prevent progressive fibrosis in chronic kidney disease by suppressing the action of TGF-β.

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Chronic progressive kidney diseases are characterized by the accumulation of fibrotic tissue in the glomerulus and in the tubulointerstitium [1,2]. This process leads to progressive loss of kidney function and finally to kidney failure, regardless of the primary disorder [1,2]. Transforming growth factor-β (TGF-β) has been identified as an important mediator of chronic fibrosis in several disease states [2–4]. The role of TGF-β in fibrogenesis is beneficial in wound repair but deleterious in chronic fibrotic diseases. This review characterizes briefly the biology of TGF-β, summarizes recent experimental data on its role in kidney diseases, and outlines potential therapeutic strategies to target TGF overexpression in disease.

## Molecular biology

Transforming growth factor-β belongs to a family of multifunctional cytokines that are dimeric proteins with a molecular weight of approximately 25 kD [5•,6]. Three isoforms (TGF-$\beta_1$, -$\beta_2$, and -$\beta_3$) located on different chromosomes have been identified in mammalian species [5•,6]. Cleavage from a larger precursor molecule is necessary for TGF-β to exert biologic activity in tissues [5•,6]. Three different TGF-β receptors have been identified. Recent data suggest that the type I and type II receptors are involved in signal transduction, whereas the type III receptor may serve as a TGF-β–binding reservoir [6].

Transforming growth factor-β plays a major biologic role in the regulation of cellular proliferation and growth, extracellular matrix synthesis, chemoattraction, angiogenesis, and in the induction of cytokines or their receptors [2–4,5•,6]. TGF-β can induce its own synthesis by cells exposed to it [7,8]. This property of autoinduction may be central to its continued overexpression in chronic fibrosis. TGF-β profoundly alters three processes involved in extracellular matrix deposition: 1) it induces synthesis of numerous extracellular matrix proteins; 2) it decreases matrix degradation by down-regulation of proteases and induction of protease inhibitors; and 3) it enhances the expression of integrins on the cell surface, which facilitates the deposition of matrix [5•]. These properties

**Abbreviations**

ACE—angiotensin-converting enzyme; ATS—antithymocyte antibodies; TGF-β—transforming growth factor-β.



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are of major importance in normal wound healing. When normal repair is complete TGF-β expression returns to normal. In contrast, chronic fibrosis is characterized by continuous TGF-β overexpression [9••].

## Acute renal injury

The basic studies on the role of TGF-β in glomerular injury and repair were performed in a rat model of immune-mediated, mesangioproliferative glomerulonephritis induced by antithymocyte antibodies (ATS) [10–14]. ATS recognizes a thy-1–like antigen on the mesangial cell surface and induces complement-dependent mesangial cell lysis [11]. The subsequent mesangial cell proliferation and glomerular extracellular matrix accumulation resemble the changes observed in mesangioproliferative glomerulonephritis in humans except these are self-limiting and normalize within several months.

Using this model, we demonstrated that TGF-β expression increases in parallel with extracellular matrix deposition [10]. Administration of TGF-β–neutralizing antibodies *in vivo* prevented extracellular matrix accumulation thus identifying TGF-β as a key mediator of fibrosis in this model [12].

The fibrogenic effects of TGF-β were shown to be due to three actions. First, TGF-β induced the synthesis of the extracellular matrix components that accumulate in glomerulosclerosis [10,12]. Second, TGF-β decreased the action of the plasmin protease system, which is thought to be important in extracellular matrix turnover [13]. TGF-β decreases the action of plasmin by depressing synthesis of the enzyme needed to generate plasmin and plasminogen activator, and simultaneously increases synthesis of its inhibitor plasminogen activator inhibitor type 1 [13]. Third, synthesis of β1 integrins, which play an important role in extracellular matrix assembly, was increased in ATS-induced glomerulonephritis following elevated TGF-β expression [14]. Interestingly, the addition of TGF-β to normal glomeruli in culture also increased the synthesis of matrix components, inhibited the plasmin protease system, and upregulated the expression of β1 integrins on the cells' surface [10,13,14].

Increased TGF-β expression has also been reported in mesangioproliferative glomerulonephritis induced by Habu snake venom, in animal models

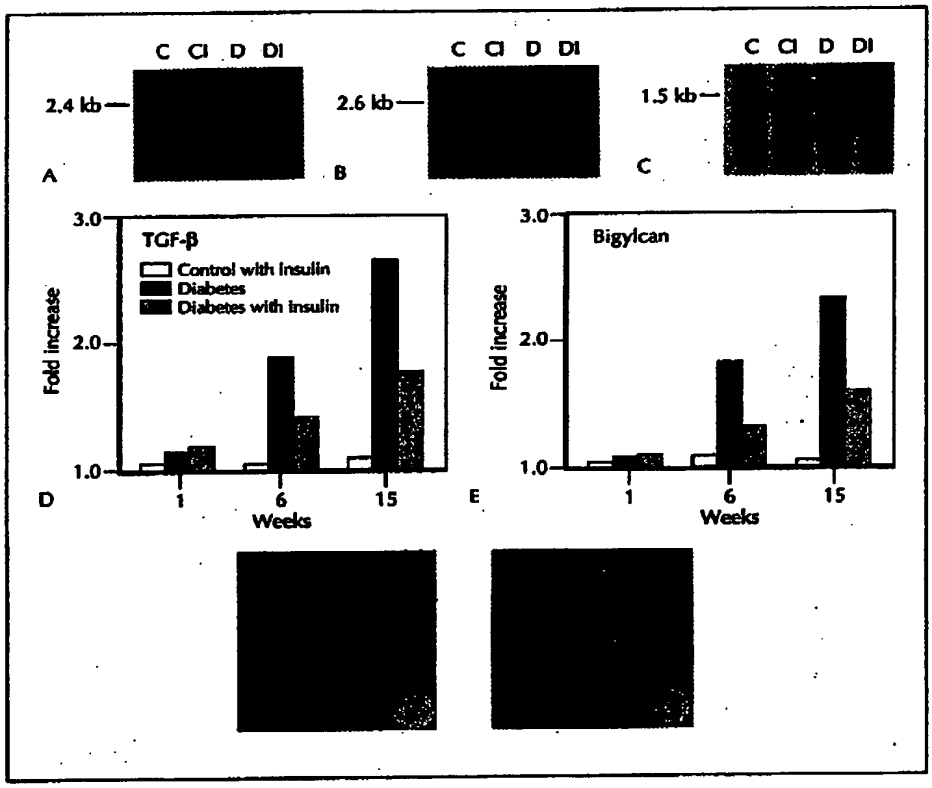


**Fig. 1.** Glomerular transforming growth factor-β (TGF-β) expression in streptozocin-induced diabetes in the rat. TGF-β (*A*), biglycan (*B*), and the control enzyme glyceraldehyde-3-phosphate dehydrogenase (*C*) messenger RNA expression in glomeruli as detected by Northern blot hybridization at 15 weeks after induction of diabetes. Relative increases of TGF-β (*D*) and biglycan (*E*) messenger RNA in the time course of diabetes are diagrammed. Glomerular TGF-β protein expression is shown by immunofluorescence in control rats (*F*) versus in untreated rats with diabetes 15 weeks after induction of diabetes (*G*). C—control rats; Cl—control rats treated with insulin; D—rats with diabetes; Dl—rats with diabetes treated with insulin. (*From* Yamamoto *et al.* [24••]; with permission.)

of crescentic nephritis induced by antiglomerular basement membrane antibodies, and in acute tubulointerstitial injury (puromycin aminonucleoside nephrosis) [15–17]. In all cases, TGF-β expression was accompanied by increased matrix protein deposition. In crescentic nephritis, urinary TGF-β excretion was correlated with the degree of glomerular sclerosis, suggesting that urinary TGF-β might be a useful indicator in progressive renal disease [18]. Recently, TGF-β upregulation was observed following unilateral ureteral ligation in a rat model of obstructive nephropathy [19]. Together, these studies show that the kidney responds to injury with increased TGF-β expression that, in turn, causes fibrosis.

## Chronic renal disease

Early studies in acute models of renal injury pointed to a key role of TGF-β induction in renal disease. The question of whether or not sustained TGF-β expression played a role in chronic progressive kidney disease, however, remained unaddressed. We answered this question by the induction of chronic renal disease using two injections of ATS [9••]. Two consecutive injections of ATS given 1 week apart led to progressive glomerular and tubulointerstitial sclerosis accompanied by increasing proteinuria [9••]. In the one-injection ATS model TGF-β expression normalized after several weeks, but rats given two ATS injections showed progressively increased glomerular TGF-β expression that with time involved the tubulointerstitium [9••,10]. Histologic changes closely resembled those seen during the development of severe renal fibrosis in humans [9••]. Both TGF-β expression and TGF-β receptors were increased in another model of chronic renal disease induced by doxorubicin hydrochloride (adriamycin model) [20].

Repeated injections of puromycin aminonucleoside also led to chronic renal fibrosis [21]. When fibrosis was apparent in this disease model, glomerular TGF-β messenger RNA expression was 15 times elevated compared with control animals [21]. Platelet-derived growth factor and basic fibroblast growth factor expression were increased, but to a lesser extent [21]. Because TGF-β is a potent inducer of platelet-derived growth factor protein and receptor expression, it likely enhances the effects of other growth factors in chronic progressive kidney disease [22].

Insulin-dependent diabetes mellitus can be induced in rats by a bolus injection of streptozocin [23] and the diabetic complications of retinopathy and nephropathy develop within months. In humans, diabetic nephropathy is the leading cause of renal failure and is characterized by progressive diffuse glomerulosclerosis. We hypothesized that TGF-β is involved in this process. Progressive increases in TGF-β messenger RNA expression were seen in glomeruli obtained from diabetic rats as early as 6 weeks after induction of disease (Fig. 1) [24••]. This increase paralleled deposition of fibronectin and tenascin, two important components of extracellular matrix. It was also recently reported that glomerular collagen synthesis is upregulated by TGF-β in diabetic rats [25].

An important experiment confirmed the ability of TGF-β alone to cause glomerulosclerosis. Isaka *et al.* [26••] injected liposomes containing TGF-β complementary DNA into the left renal artery of otherwise normal rats. This was followed by increased expression of the TGF-β protein and severe glomerulosclerosis within 7 days. The right kidney was completely unaffected by this procedure, which clearly demonstrates that TGF-β alone is a potent inducer of extracellular matrix accumulation in glomeruli.

**Table 1.** Elevated transforming growth factor-β expression in experimental renal disease

| Study | Model | Disease |
|---|---|---|
| Yamamoto *et al.* [9••] | Animal (chronic) | Glomerulosclerosis (induced by repeated antithymocyte antibody injections) |
| Okuda *et al.* [10] | Animal (acute) | Antithymocyte antibody–induced glomerulonephritis |
| Barnes and Abboud [15] | Animal (acute) | Mesangioproliferative glomerulonephritis (Habu snake venom) |
| Coimbra *et al.* [16] | Animal (acute) | Antiglomerular basement membrane nephritis |
| Jones *et al.* [17] | Animal (acute) | Puromycin aminonucleoside nephrosis |
| Kaneto *et al.* [19] | Animal (acute) | Unilateral ureteral ligation |
| Tamaki *et al.* [20] | Animal (chronic) | Glomerulosclerosis (induced by adriamycin injection) |
| Nakamura *et al.* [21] | Animal (chronic) | Glomerulosclerosis (induced by repeated puromycin aminonucleoside injections) |
| Yamamoto *et al.* [24••] | Animal (chronic) | Diabetic nephropathy |
| Junaid *et al.* [32] | Animal (chronic) | Remnant kidney (subtotal nephrectomy) |

All of these studies show that TGF-β is a major mediator of chronic glomerular and tubulointerstitial fibrosis in progressive kidney diseases. Investigations using numerous other models of organ fibrosis in liver, lung, skin, arteries, and central nervous system confirm that TGF-β plays a role in chronic fibrosis [2,3].

## Human kidney disease

Recent reports show that the experimental data presented thus far are relevant to progressive kidney disease in humans. Kidney biopsies from patients with overt diabetic nephropathy showed significantly increased glomerular TGF-β expression compared both with normal kidney tissue and with material obtained from patients with minimal change disease and thin basement membrane disease; these diseases do not progress to sclerosis [24••]. The quantity of staining closely correlated with the severity of glomerulosclerosis. Table 1 lists renal disease models in which elevated TGF-β expression has been found.

## The renin-angiotensin system and transforming growth factor-β

Angiotensin II is thought to damage kidneys by increasing glomerular hydrostatic capillary and systemic blood pressure [27]. The beneficial effects of angiotensin-converting enzyme (ACE) inhibitors in slowing disease progression support this point of view [28]. Recent studies on the interaction of the renin-angiotensin system and TGF-β suggest that another pathway exists.

Gibbons *et al.* [29] showed that angiotensin II induces TGF-β messenger RNA expression in cultured vascular smooth muscle cells. Wolf *et al.* [30] confirmed these findings in proximal tubular cells *in vitro*, and both studies demonstrated that angiotensin II–induced TGF-β expression leads to hypertrophic growth. Using cultured rat mesangial cells, we recently reported that angiotensin II stimulates TGF-β expression and that TGF-β then induces increased synthesis of matrix proteins [31••]. *In vivo* subcutaneous infusion of angiotensin II led to increased glomerular TGF-β gene expression within 1 week. This increase was associated with the induction of collagen type I messenger RNA and glomerular matrix accumulation [31••]. Interestingly, in unilateral ureteral ligation and in the remnant kidney model in the rat, TGF-β messenger RNA expression was significantly blunted by *in vivo* administration of an ACE inhibitor or an angiotensin II receptor antagonist, respectively [19,32]. These data suggest that angiotensin II directly induces TGF-β, which in turn induces fibrotic changes.

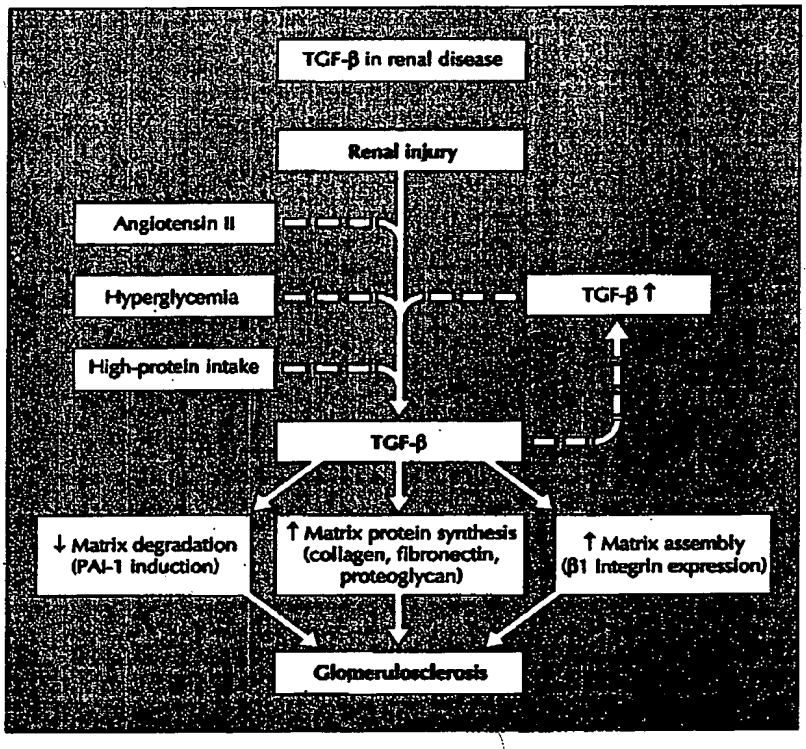


**Fig. 2.** The role of transforming growth factor-β (TGF-β) overexpression in the pathogenesis of glomerulosclerosis and potential targets of treatment. PAI—plasminogen activator inhibitor.

## Therapeutic strategies targeting transforming growth factor-β overexpression

Three clear strategies exist to decrease TGF-β expression *in vivo*: inhibition of angiotensin II, low-protein diet, and direct antagonists of TGF-β. In addition to influencing the hemodynamic effects of angiotensin II, ACE inhibitors and angiotensin II receptor antagonists may also inhibit the angiotensin II–induced cascade of both increased TGF-β expression and increased matrix protein synthesis and deposition. It will be interesting to determine whether or not these drugs lead to a reduction in TGF-β expression *in vivo*.

Another treatment slowing the progression of some renal diseases is a low-protein diet, which decreases glomerular hyperfiltration and reduces plasma renin activity [33–35]. We showed that dietary protein restriction downregulated TGF-β expression in ATS-induced glomerulonephritis [36]. A decrease in the activity of the renin-angiotensin system may have contributed to this effect. Recent data from our laboratory suggest that restriction of the amino acid L-arginine may be the key factor in low-protein diet because low dietary L-arginine also downregulates TGF-β expression in this model, even when total dietary protein intake is normal [37]. Metabolites of L-arginine are involved in tissue injury (nitric oxide), cell proliferation (polyamines), and collagen synthesis (L-proline), processes ongoing in many renal diseases.

A number of antagonists of TGF-β are possible. Soluble receptors, antisense oligonucleotides, and *humanized* antibodies are three possibilities. Another potential therapeutic antagonist is the proteoglycan decorin, which binds and neutralizes TGF-β *in vitro* [38]. Decorin is a normal extracellular matrix component that can be manufactured in large quantities using recombinant technology. In ATS-induced glomerulonephritis, the *in vivo* administration of decorin significantly blunted extracellular matrix accumulation within the glomerulus and was as potent as an injection of TGF-β neutralizing antibody [39].

The mechanism of decorin's antagonism of TGF-β and its efficacy in progressive kidney fibrosis are currently being studied. A study of the bleomycin model of pulmonary lung fibrosis in the rat found decreased decorin expression in the initial phases of the disease followed by increased expression during the reparative phase [40]. It is possible that decorin plays a role in the termination of normal wound repair by blocking the TGF-β effects and by interrupting TGF-β autoinduction. Progressive fibrosis might then represent a state of decorin deficiency.

## Conclusions

The experimental and human studies reviewed show that TGF-β plays a causative role in glomerular and tubulointerstitial fibrosis; potential mechanisms are summarized in Figure 2. Antagonists of the action of TGF-β are therefore very promising therapeutic agents. ACE inhibitors and low-protein diets may slow the progressive course of some kidney diseases by suppressing TGF-β expression. The TGF-β binding proteoglycan decorin may become an antifibrotic drug for the treatment of chronic progressive kidney failure.

## Acknowledgment

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tion. All immunoreactive IRGT present in adipose tissue was detected in the isolated adipocyte fraction (Fig. 4).

We conclude that the level of expression of the IRGT in endothelial cells is insignificant compared with that observed in myocytes and adipocytes. Using three different antibodies we have consistently observed negligible labelling of endothelial cells in skeletal muscle, heart and adipose tissue of five non-insulin-treated and five insulin-treated rats, except for an occasional, spurious reaction with 1F8. We observed other faulty reactions with 1F8, for instance in nuclei of all cell types and in the lumen of myocyte sarcoplasmic reticulum. These observations are at odds with the apparent cytosolic orientation of the epitope for 1F8. These spurious reactions, including those observed in endothelial cells, were not consistently present in 1F8-labelled tissue sections and never in the immunoblotting experiments. Moreover, this aberrant labelling pattern was never observed using polyclonal antibodies. We were able to compete out both 1F8 and the polyclonal antibody immunolabelling of cells when the antibodies were preincubated with a 10-fold molar excess of the IRGT C-terminal peptide. In fact this was even evident with respect to the endothelial cell and nuclear staining of 1F8. This might indicate that the spurious labelling observed with 1F8 is due to weak cross reaction with other than IRGT molecule(s). Most probably this can only result in low stringent binding, so that minor variations in the reaction conditions can explain why the cross reaction is only sometimes manifest in immunolabelled preparations, and why these spurious 1F8 reactions are not reported from immunoprecipitation[10] or immunoblotting (Fig. 4) studies. These observations suggest that localization of the IRGT using only 1F8 may be difficult to interpret and we suspect that the previous data[15] may largely reflect the aberrant labelling pattern of the monoclonal antibody.

Our data indicate that it is the permeability of the myocyte or adipocyte membrane that limits entry of glucose into these cells. Previous studies have shown quite convincingly that in cardiac muscle there is no significant difference in transcapillary flux of L- compared with D-glucose[17,18]. Thus, the existence of a transporter to facilitate the movement of glucose out of the endothelium, at least in cardiac muscle, would seem to be redundant. We conclude that, in muscle and adipose tissue, the IRGT is primarily expressed in myocytes and adipocytes, respectively. In these cells the transporter is enriched in the trans Golgi reticulum and in small vesicles and tubules scattered throughout the cytoplasm. These sites of IRGT seem to be in an insulin-regulated equilibrium with the plasma membrane. □

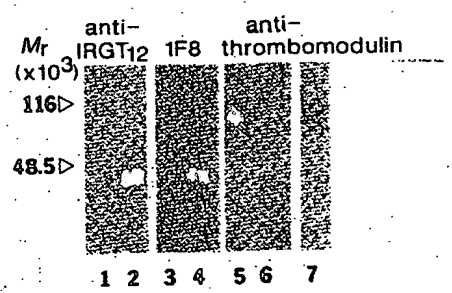


FIG. 4 Distribution of the IRGT (lanes 1–4) and thrombomodulin (lanes 5–7) in endothelial cells (lanes 1, 3 and 5) and adipocytes (lanes 2, 4 and 6) isolated from rat epididymal fat pads. Aliquots (10 μg total protein) of the homogenates were immunoblotted with either anti-$IRGT_{12}$ (lanes 1, 2), 1F8 (lanes 3, 4) or anti-thrombomodulin[21] (lanes 5, 6, 7). An aliquot of purified rat thrombomodulin was also immunoblotted as a reference (lane 7). Relative molecular weights ($M_r$) are indicated on the left.

METHODS. Epididymal fat pads were dissected from male rats (150 g) and digested with collagenase for 45 min at 37 °C as previously described[11,12]. Endothelial cells were prepared essentially as described[20]. Following collagenase digestion, cells were passed through a 250-μm pore nylon filter. The fat cells were allowed to float to the surface and removed. The remaining filtrate was centrifuged at 400*g* (5 min). The pellet was washed with Krebs Ringer buffer and passed through a 20-μm pore nylon filter. The clumps of endothelium that remained attached to the filter were washed in buffer and recentrifuged. Inspection of this fraction by light microscopy revealed an identical morphology to that reported previously for microvascular endothelium with little contamination from cells of other origin[20]. From 50 rats we isolated ~250 μg of endothelial cell protein.

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# Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β1

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**GLOMERULONEPHRITIS is an inflammation of the kidney characterized by the accumulation of extracellular matrix within the damaged glomeruli[1–4], impaired filtration and proteinuria. In its progressive form, the disease destroys kidney function leading to uraemia and death, unless dialysis therapy or kidney transplantation is available. The pathogenesis of glomerulonephritis is incompletely understood, but the eliciting factor is thought often to be an immunological injury to mesangial and/or other resident cells in the glomeruli[5,6]. We have used an animal model of acute mesangial proliferative glomerulonephritis[7,8] to show that this disease is associated with increased production and activity of transforming growth factor β1 (TGF-β1)[9], an inducer of extracellular matrix production[10–17]. Here we report that administration of anti-TGF-β1 at the time of induction of the glomerular disease suppresses the increased production of extracellular matrix and dramatically attenuates histological manifestations of the disease. These results provide direct evidence for a causal role of TGF-β1 in the pathogenesis of the experimental disease and suggest a new approach to the therapy of glomerulonephritis.**

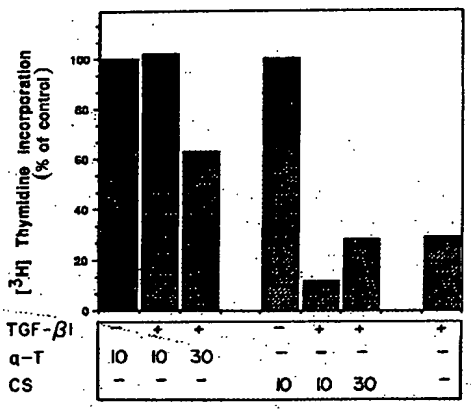


FIG. 1 Inhibition of TGF-β1 activity by antibodies. A mink lung epithelial cell assay that measures growth inhibition by TGF-β (ref. 30) was used to assay the ability of anti-TGF-β antiserum to neutralize its activity. Dilutions of 1:10 (10) and 1:30 (30) of anti-TGF-β1 serum (a-T) inhibited the action of TGF-β1 (TGF-β1) added to the cultured cells. Control antiserum (CS) had no effect (+, added; −, not added).
METHODS. Anti-TGF-β1 serum was prepared against a synthetic peptide from residues 78–109 (ref. 18) of human mature TGF-β1. The peptide was synthesized in an Applied Biosystems solid phase peptide synthesizer and purified by HPLC. The rabbit was immunized subcutaneously with 2 mg per injection of the peptide which was mixed with 0.5 mg methylated BSA[31] and emulsified in Freund's complete adjuvant. The injections were generally given 4 weeks apart and the rabbit was bled about 1 week after the second and every successive injection. The antisera used in this work had a titre (50% binding) of 1:6,000 in radioimmunoassay, bound to TGF-β1 in immunoblots and inhibited the induction of proteoglycan synthesis caused by TGF-β1 in cultured mesangial cells (results not shown). Growth inhibition was assayed by adding to mink lung epithelial cell cultures 3 ng ml$^{-1}$ human platelet TGF-β1 (Calbiochem) which had been preincubated with dilutions of 1:10 or 1:30 of heat-inactivated (56 °C, 30 min) anti-TGF-β1 serum or with identical dilutions of a control antiserum prepared against an unrelated peptide (from the cytoplasmic domain of the $\alpha_4$-subunit of an integrin), followed by an assay for [$^3$H]thymidine incorporation[30].

Glomerular mesangial cells express a Thy-1.1 epitope on their surface[7]. Injection of antithymocyte serum into an animal produces a dose- and complement-dependent selective injury to mesangial cells resulting in acute mesangial proliferative glomerulonephritis[7,8]. The glomerular lesion is characterized by expansion of the mesangial matrix and hypercellularity, resembling the morphological features of human mesangial proliferative glomerulonephritis.

Induction of glomerulonephritis in rats with a single injection of antithymocyte serum was followed by treatment with an injection of either anti-TGF-β1 or control sera. The anti-TGF-β1 serum was prepared by immunizing a rabbit with a synthetic peptide containing residues 78–109 from human mature TGF-β1; the cyclized form of this peptide elicits an antiserum capable of inhibiting binding of TGF-β1 to cells[18]. The specificity of the antiserum was established as described in the legend of Fig. 1 and it can neutralize the activity of purified TGF-β1 in a bioassay (Fig. 1).

The effects of the anti-TGF-β antiserum and control sera on the glomerulonephritis model were evaluated by testing 10 animals in each group in three different experiments. We used histological evaluation of glomerular extracellular matrix accumulation as a measure of disease activity, because it is a characteristic feature of acute glomerulonephritis[1–4].

Figure 2 shows a comparison of the microscopic appearance of representative glomeruli, and measurement of the glomerular extracellular matrix is shown in Fig. 3. The glomeruli contain much less extracellular matrix material in the anti-TGF-β1-treated groups than in the groups receiving control serum. Immunofluorescent staining of the tissues with antibodies

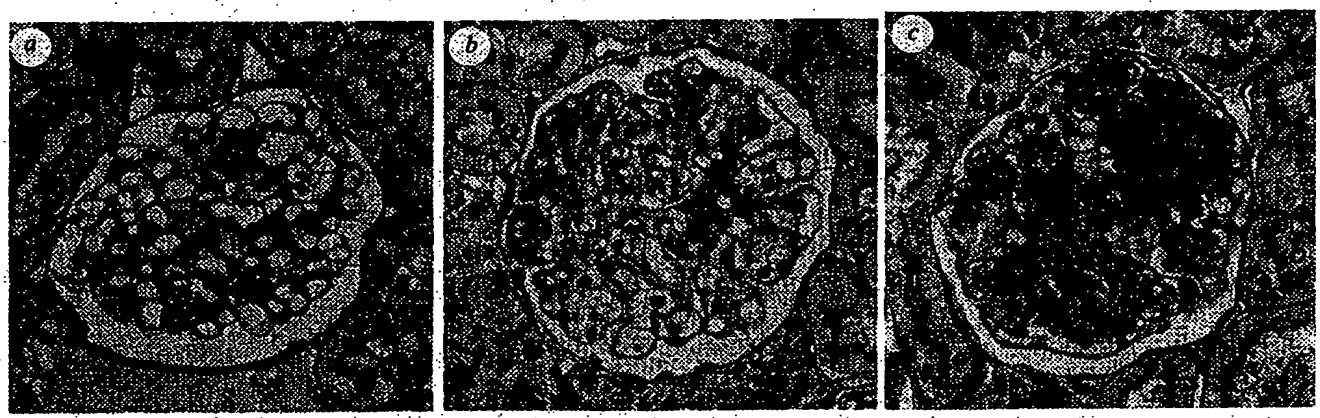


FIG. 2 Histological analysis showing pathological changes in glomeruli of glomerulonephritic kidneys. Micrographs showing periodic acid-Schiff staining of glomeruli. *a*, Staining of the basement membrane and extracellular matrix in a normal glomerulus. *b*, From a rat on day 7 after the injection of antithymocyte serum. This rat received injections of 1 ml rabbit anti-TGF-β1 on six successive days, starting on the day of the antithymocyte injection. *c*, From an animal that received control serum under a similar regimen. A striking increase in extracellular matrix is seen as the reddish-pink amorphous material filling most of the glomerulus in *c*; *b* shows a clear effect of anti-TGF-β1 in preventing the increase in glomerular extracellular matrix that occurs on day 7 after injection of antithymocyte serum. Magnification, ×500.
METHODS. Antithymocyte serum was produced by immunizing New Zealand white rabbits with $1\times10^6$ rat thymocytes in complete Freund's adjuvant, followed by boosting with $1\times10^6$ thymocytes given intravenously 2 and 4 weeks later[9]. The serum was absorbed three times each with packed rat erythrocytes and rat liver powder to remove nonspecific reactivity. Glomerulonephritis was induced in Sprague Dawley rats (4–6 weeks old) by intravenous (i.v.) administration of 1 ml antithymocyte serum per 100 g body weight[9]. The anti-TGF-β1 serum and the two rabbit sera used as controls were also administered i.v. All sera were heat-inactivated at 56 °C for 30 min before injection. The extent of glomerular injury was evaluated by performing glomerular cell counts from 30 randomly selected glomeruli from 10 normal animals and nephritic animals in each group on days 4 and 7. Normal rat glomeruli contained 52 ± 14 cells. On day 4 there was a decreased number of cells (35 ± 11) as a result of cell lysis by the antithymocyte antibody, whereas an increased number of cells was seen on day 7 (68 ± 15). Values are mean ± s.d. The changes in cellularity were the same in the anti-TGF-β1 treatment and control groups.

against fibronectin, laminin and type IV collagen showed that the pathological matrix, as well as the normal matrix, contained each of these proteins (results not shown).

In a previous study we found that the injured glomeruli in culture produce more extracellular matrix components than do cells from normal glomeruli, and that the synthesis of two proteoglycans, biglycan and decorin, is particularly high[9]. This pattern of proteoglycan synthesis is similar to that obtained by adding TGF-β1 to cultures of rat mesangial cells from normal glomeruli[17], and antibody inhibition experiments and TGF-β assays have shown that it is caused by increased synthesis of TGF-β1 in the glomeruli[9]. We therefore used the production of proteoglycans as a bioassay that would reflect the activity of TGF-β and its influence on extracellular matrix synthesis in our disease model. Such an analysis showed that proteoglycan production by glomerular cells was suppressed to a near normal rate by anti-TGF-β1 (Fig. 4*a*). Scans of the gel bands (Fig. 4*a*) and other similar experiments indicated that the suppression of this measure of the disease process was about 60% on day 4 and 80% on day 7 after injection. These results show that the glomerular disease was substantially attenuated by the anti-TGF-β1 treatment.

The level of TGF-β1 messenger RNA in the glomeruli of glomerulonephritis rats is higher than in normal rats and these glomeruli contain more cells producing TGF-β1 than do normal glomeruli[9]. These results indicate that increased expression of the TGF-β1 gene is correlated with injury in this model. Messenger RNA analysis of the anti-TGF-β1-treated and control glomeruli on the current study revealed equally raised levels of TGF-β1 mRNA in both groups of rats (Fig. 4*b*). A similar increase in the number of TGF-β1-positive cells was also seen in the treated and control animals (results not shown). These results indicate that anti-TGF-β1 did not interfere with induction of glomerular injury, as reflected by the increased synthesis of TGF-β1 mRNA and TGF-β1 protein in glomeruli. The cells responsible for the increased TGF-β1 expression have not been identified, but they could be proliferating mesangial cells and/or infiltrating monocyte/macrophages.

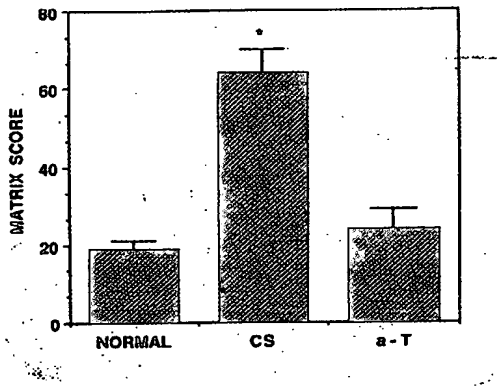


FIG. 3 Quantitation of extracellular matrix accumulation in nephritic glomeruli. Asterisk denotes $P < 0.001$, for glomerulonephritic rats treated with control serum (CS) compared with anti-TGF-β1 (a-T) on day 7 of glomerulonephritis. Normal (normal) rats were included for comparison. Values are mean ±s.d. METHODS. To quantitate mesangial matrix all sections were coded and read by an observer unaware of the experimental protocol applied. Thirty glomeruli (80–100 μm in diameter) were selected at random in sections prepared from normal rats or from anti-TGF-β1 rats and control-treated rats on day 7 of glomerulonephritis. The degree of glomerular matrix expansion was determined as the percentage of each glomerulus occupied by mesangial matrix by using a published method[31]. Differences between groups in matrix scores were analysed by *t*-test. Two types of control sera were used: a normal rabbit serum, and a rabbit antiserum prepared against an unrelated peptide. Neither had any effect on the glomerulonephritis, and the results were pooled for the figure.

Our findings establish a central role for TGF-β in the pathogenesis of acute mesangial proliferative glomerulonephritis. TGF-β can greatly stimulate the production of extracellular matrix components by various kinds of cells[10–16], including the production of the two proteoglycans[20,21] we have used as markers of TGF-β activity in this and earlier studies[9,17]. Because of this activity, TGF-β has been suspected to have a role in fibrotic diseases resulting from various chronic disease processes[22,23]. Whereas earlier results have provided correlative evidence for a role of TGF-β in glomerulonephritis, the antibody-inhibition data presented here establish that TGF-β has a direct, causal role, at least in the experimental glomerulonephritis model we have used. The type of TGF-β responsible for the increased production of extracellular matrix in our model

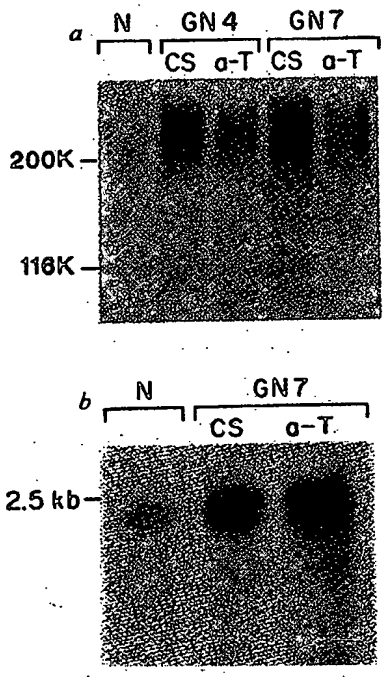


FIG. 4 *a*, Proteoglycan synthesis by glomeruli from glomerulonephritic rats treated with anti-TGF-β1. CS, glomerulonephritic rats treated with control serum; a-T, glomerulonephritic rats treated with rabbit anti-TGF-β1 on days 4 (GN 4) and 7 (GN 7) after injection of antithymocyte serum. The control lane (N) shows proteoglycan production in glomeruli from normal rat kidney. *b*, Northern blotting of TGF-β1 mRNA in glomeruli isolated from the kidneys of normal and treated glomerulonephritic rats. Scanning of the bands showed a fivefold increase, relative to normal controls, in TGF-β1 mRNA in both anti-TGF-β1 (a-T) treatment and control (CS) groups on day 7. The control lane (N) shows TGF-β1 mRNA in glomeruli from normal rat kidney.
METHODS. *a*, Glomeruli were isolated 4 and 7 days after an antithymocyte serum injection that had been followed by treatments similar to those described in the legend of Fig. 3, and placed in culture[9]. Proteoglycan synthesis was examined by labelling the cultures with $^{35}SO_4$ followed by analysis of the secreted products by SDS-PAGE and autoradiography as described[9,17]. Markers were myosin and β-galactosidase with relative molecular masses ($M_r$) of 200,000 and 116,000, respectively ($M_r \times 10^{-3}$). *b*, Total RNA was prepared by lysis of isolated glomeruli in guanidine isothiocyanate and ultracentrifugation of lysate on a caesium chloride cushion[33]. Samples of 10 μg were electrophoresed in a 2.2 M formaldehyde/1% agarose gel and transferred to a nitrocellulose membrane. The membranes were prehybridized for 5 h at 37 °C in 5 × SSC, 5 × Denhardt's solution, 0.1 mg ml$^{-1}$ salmon sperm DNA, 0.1% SDS and 50% formamide. A porcine TGF-β1 complementary DNA probe[34] and a rat glyceraldehyde 3-phosphate dehydrogenase cDNA probe[35] were labelled with [$^{32}$P]CTP by the random primer method and hybridized at 42 °C for 15 min. The TGF-β1 mRNA result is shown; the amount of the dehydrogenase mRNA was equal in all samples and is not shown.

is likely to be TGF-β1 because the antiserum was prepared against a peptide from this TGF-β. Because the amino-acid sequence of the various TGF-βs are similar[14,24], however, the antiserum could also have affected other types of TGF-β.

The mechanism of TGF-β1 action is likely to be the extremely strong stimulation of extracellular matrix synthesis, manifested as a marked induction of proteoglycan synthesis by TGF-β1 in cultural mesangial cells and of proteoglycan, fibronectin and other matrix molecules in cultured glomerular epithelial cells[17,25]. The ability of TGF-β1 to suppress the expression of proteases and stimulate the expression of protease inhibitors[26,27] may also contribute to the accumulation of extracellular matrix.

We have used antithymocyte serum to produce acute mesangial matrix expansion *in vivo*. Although the aetiological mechanism in man is not known, accumulation of mesangial matrix is also a prominent and important pathological feature of human mesangial proliferative glomerulonephritis[28] and diabetic nephropathy[32]. The impressive suppression of the experimental disease achieved with anti-TGF-β1 treatment indicates the importance of TGF-β in regulating extracellular matrix in glomerulonephritis. This suggests that TGF-β may have a similar role in human glomerular diseases, and perhaps other diseases in which fibrosis is a factor. □

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# Voltage-dependent $InsP_3$-insensitive calcium channels in membranes of pancreatic endoplasmic reticulum vesicles

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**STIMULUS–SECRETION coupling in exocrine glands involves $Ca^{2+}$ release from intracellular stores[1,2]. In endoplasmic reticulum vesicle preparations from rat exocrine pancreas, an inositol 1,4,5-trisphosphate($InsP_3$)-sensitive, as well as an $InsP_3$-insensitive, $Ca^{2+}$ pool has been characterized[3]. But $Ca^{2+}$ channels in the endoplasmic reticulum of rat exocrine pancreas have not been demonstrated at the level of single-channel current. We have now used the patch-clamp technique on endoplasmic reticulum vesicles fused by means of the dehydration–rehydration method[4–6]. In excised patches, single $Ba^{2+}$- and $Ca^{2+}$-selective channels were recorded. The channel activity was markedly voltage-dependent. Caffeine increased channel open-state probability, whereas ruthenium red and $Cd^{2+}$ blocked single-channel currents. Ryanodine, nifedipine and heparin had no effect on channel activity. The channel activity was not dependent on the free $Ca^{2+}$ concentration, the presence of $InsP_3$, or pH. We conclude that this calcium channel mediates $Ca^{2+}$ release from an intracellular store through an $InsP_3$-insensitive mechanism.**

The endoplasmic reticulum (ER) of rat exocrine pancreas is known to play a central part in the regulation of cytoplasmic free $Ca^{2+}$ concentration[1,7,8]. To investigate whether $Ca^{2+}$ channels are involved in the $Ca^{2+}$ release mechanisms, isolated ER vesicles were fused to giant vesicles (10–100 μm) by the dehydration–rehydration method[4–6]. These giant ER vesicles were used for studies with the patch-clamp technique. To avoid current fluctuations from the large conductance anion channel[5], $Cl^-$ was replaced by HEPES buffer in both bath and pipette solutions. With $K^+$ buffer (75 mM KOH, 280 mM HEPES buffer, pH 7.1) in the pipette and $Ba^{2+}$ buffer (50 mM $Ba(OH)_2$, 280 mM HEPES buffer, pH 7.3) in the bath, single-channel currents carried by $Ba^{2+}$ ions flowing from the bath into the pipette were recorded in 87% of stable seals. The mean channel conductance was $47 \pm 3$ pS ($n = 19$). Replacement of $Ba^{2+}$ by $Ca^{2+}$ showed that the channel is also permeable to $Ca^{2+}$ ions. A single membrane patch contained 1–4 (usually 1 or 2) channels.

Typical single-channel current traces are shown in Fig. 1*a*. The conductance of this channel determined by linear regression was 41 pS (Fig. 1*b*). From the extrapolated reversal potential of −31 mV, a permeability ratio $P_{Ba^{2+}}/P_{K^+}$ of ~15:1 is estimated. The channel activity displayed a distinct voltage-dependence (Fig. 1*a* and *c*). Positive clamp voltages (+10 to +20 mV; sign refers to bath side) increased channel open-state probability, whereas negative potentials led to channel inactivation. In addition to the full current amplitude, sublevels of conductance occurred (Fig. 1*a*). Frequency, duration and magnitude of the sublevels varied in different experiments. As well as the voltage-dependent inactivation, in most experiments a spontaneous run-down of channel activity occurred with time; channel activity could be restored by applying short voltage pulses (±40 mV).

To further characterize the channel, several potential $Ca^{2+}$ channel activators and blockers were tested. Ryanodine[9,10] (pipette 200 μM, $n = 11$; bath 50 μM, $n = 5$), which is known to arrest the $Ca^{2+}$ release channel from skeletal muscle sarcoplasmic reticulum (SR) on a sublevel of conductance, had no effect on $Ca^{2+}$ channels in pancreatic ER membranes. Neither heparin (100 μg $ml^{-1}$, $n = 7$), an inhibitor, nor $InsP_3$ (10–50 μM, $n = 10$), an activator of $InsP_3$-sensitive $Ca^{2+}$ channels in smooth muscle SR[11], nor nifedipine (10–100 μM, $n = 9$), a blocker of voltage-dependent dihydropyridine-sensitive L-type calcium channels[12], had any detectable effects on either side of the membrane patch. The $Ca^{2+}$ channel in fused pancreatic ER membranes, therefore,

# Transforming growth factor-β and the pathogenesis of glomerular diseases

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Transforming growth factor-β (TGF-β) is a cytokine that is important in embryogenesis, development, carcinogenesis, and tissue repair. TGF-β is unique among cytokines in its widespread actions on the regulation of extracellular matrix. In a model of acute mesangial proliferative glomerulonephritis, it was shown that overproduction of TGF-β is the cause of pathologic matrix accumulation in the nephritic glomeruli. TGF-β acted to increase matrix production, inhibit matrix degradation, and modulate matrix receptors in the glomerulonephritic rats. Elevated expression of TGF-β was also found in other experimental glomerular diseases, including diabetic nephropathy. Studies of humans with glomerulonephritis and diabetic nephropathy also strongly implicated TGF-β in the pathogenesis of glomerular matrix build-up. Recently, the proteoglycan decorin was shown to neutralize TGF-β. When injected into glomerulonephritic rats, decorin markedly suppressed pathologic matrix deposition in the glomeruli. Thus, decorin offers hope as a treatment for progressive kidney diseases caused by the overproduction of TGF-β.

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Extracellular matrix is composed of a mixture of interacting glycoproteins, collagens, and proteoglycans. Cells attach directly to the matrix by specific receptors, called *integrins*, that connect via the cytoskeleton to the nucleus, providing a direct line of communication between the cell and the outside world. Cell attachment to matrix is essential for differentiation and the formation of specific tissues and is also a key event in carcinogenesis and tissue repair. Recent evidence shows that essential biologic events, such as embryogenesis, development, and tissue repair, are regulated by the actions of peptide factors called *cytokines* (or growth factors). Transforming growth factor-β (TGF-β) is a prototypical cytokine, and one of the major actions of TGF-β is regulation of extracellular matrix deposition. TGF-β is unique among cytokines in its actions to: 1) stimulate the synthesis of matrix proteins by cells, 2) inhibit the proteases that degrade matrix, and 3) modulate integrins on the cell surface to allow greater binding to matrix. These three actions strongly promote deposition and accumulation of matrix in tissues. Excessive deposition of extracellular matrix is the central feature of fibrotic diseases, such as glomerulonephritis, diabetic nephropathy, cirrhosis, and pulmonary fibrosis, that destroy organ function. A growing body of evidence strongly implicates TGF-β as the cause of these fibrotic diseases [1•,2,3]. Abboud [4] has provided an excellent overview of the action of cytokines, including TGF-β, in glomerulonephritis.

## Transforming growth factor-β and cultured glomerular cells

Previously, TGF-β was shown to stimulate the production of proteoglycans by cultured rat mesangial cells, whereas other cytokines (platelet-derived growth factor [PDGF], interleukin-1, and tumor necrosis factor) had no effect on matrix synthesis [5]. In a new report, this work was extended to rat glomerular (visceral) epithelial cells [6]. TGF-β was found to induce the production of fibronectin, type IV collagen, laminin, and biglycan. Of interest, TGF-β did not alter the synthesis of heparan sulfate, another component of the glomerular basement membrane. As with the mesangial cells,

**Abbreviations**
PA—plasminogen activator; PAI—plasminogen activator inhibitor; PDGF—platelet-derived growth factor; TGF-β—transforming growth factor-β.



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TGF-β did not stimulate cell proliferation. These results indicate that TGF-β, released in the glomerulus following injury, can cause both cell types to increase production of extracellular matrix. In a related study, TGF-β was found to increase the production of proteoglycans by rabbit renal proximal tubule cells and to increase the structural integrity of the cytoskeleton [7]. These effects may be important in the regeneration of the tubule following injury.

All cells, except for contrived mutants, synthesize and release TGF-β. Mesangial cells in culture express a 2.5-kb TGF-β mRNA and constitutively secrete a substantial amount of TGF-β, mostly in the latent form [8]. TGF-β is proteolytically processed into a 25-kD mature homodimer that is secreted noncovalently bound to a portion of the precursor protein that confers biologic latency. Either acidic conditions or proteases, *eg*, plasmin, can dissociate the latency peptide and release active TGF-β. How TGF-β is activated *in vivo* is unknown. PDGF was found to not alter TGF-β expression, however, phorbol ester did stimulate mesangial cells to express TGF-β mRNA. Thus, mesangial cells were shown to produce TGF-β in a manner implicating TGF-β as an autocrine factor that is biologically active in these cells [8].

In wound repair, TGF-β and PDGF are companion cytokines with quite distinct actions. Both are released by platelets at sites of injury such as in areas of mesangial cell lysis following administration of antithymocyte serum, as employed in a popular experimental model of mesangial proliferative glomerulonephritis [9]. Under standard conditions TGF-β does not stimulate mesangial cell proliferation and has unique actions on extracellular matrix, as already described [5,10]. In contrast, PDGF is a potent mitogen for mesangial cells but does not affect matrix synthesis [5,11]. Johnson *et al.* [9] emphasized the concept that PDGF-induced mesangial cell proliferation is a key component in mesangial matrix accumulation. Although dividing cells do produce matrix and an increased number of cells necessitates more matrix, it is clear that TGF-β is capable of increasing matrix production without cell proliferation [5]. Furthermore, TGF-β may regulate PDGF production by mesangial cells. TGF-β was shown to increase expression of mRNAs of both PDGF B chain and PDGF β-receptor [12]. The increased PDGF expression was associated with increased density of cell surface PDGF receptors and stimulation of mesangial cell proliferation. This report also summarized evidence from other cell lines in which PDGF is also regulated in a complex way by TGF-β [12]. One therapeutic implication of this work is that blocking TGF-β may simultaneously prevent matrix accumulation and excessive cell proliferation in glomerulonephritis.

## Mesangial proliferative glomerulonephritis

Transforming growth factor-β has been shown to strongly induce matrix protein synthesis in cultured glomerular cells and, *in vivo*, in acute mesangial proliferative glomerulonephritis induced by injection of antithymocyte serum [5,6,13]. A particularly powerful tool for investigating this model is the ability to acutely isolate and culture nephritic glomeruli in order to perform molecular studies. The use of these techniques in glomerular disease was recently reviewed [14]. By day 4 of glomerulonephritis, there was a substantial increase in synthesis of proteoglycans and fibronectin by the nephritic glomeruli. Simultaneously, there was increased deposition of matrix in the mesangial areas. The increased matrix production correlated with increased expression of TGF-β mRNA and protein in the nephritic glomeruli. That TGF-β was causal in stimulating the matrix production was shown by the ability of TGF-β antibody to suppress matrix synthesis when added to nephritic glomeruli in culture. In a subsequent study, injection of TGF-β antibody into nephritic rats dramatically suppressed the expected increase in matrix production by the nephritic glomeruli and prevented the build-up of pathologic matrix, which is characteristic of the disease [15].

The second action of TGF-β to inhibit matrix degradation was also investigated in this model [16]. One of the proteases strongly influenced by TGF-β is the plasminogen activator/plasmin system. Plasmin is a potent protease that is best known for its activity against fibrin, but plasmin is also capable of degrading most matrix proteins and probably plays an important role in normal matrix turnover. Plasmin generation is regulated by the interaction of plasminogen activators (PAs) and plasminogen activator inhibitors (PAIs). It was found that PA activity was markedly reduced and PAI synthesis dramatically increased when TGF-β was added to normal glomeruli in culture. In the glomerulonephritic model, it was shown that prior to matrix accumulation there were striking changes in the PA/PAI system that would favor matrix deposition [16]. PA activity was dramatically decreased and PAI synthesis increased by day 3 of disease, and both returned toward normal by day 7. The increased synthesis of PAI was reflected in increased PAI deposition into the glomerular matrix, where it acts to block plasmin generation. These findings indicate that the components necessary for blocking matrix degradation by plasmin are in place early in the disease process, prior to histologic evidence of matrix accumulation. Furthermore, a causal role for TGF-β in regulating these changes was demonstrated by showing that *in vivo* administration of TGF-β antibody blocked the disease-induced deposition of PAI in the glomerular matrix.

The third action of TGF-β influencing matrix deposition is the modulation of integrins on the cell surface that mediate cell matrix contact. Integrins constitute a family of heterodimeric glycoproteins consisting of noncovalently associated α and β subunits. The synthetic profiles of integrins during the course of disease were investigated in the acute glomerulonephritis model [17]. By day 7 of disease, there was a marked increase in

synthesis and expression of the classic fibronectin receptor $\alpha_5\beta_1$, which was paralleled by a build-up of fibronectin in the expanding mesangial matrix. Other integrin receptors not involved in matrix deposition showed no change or actually decreased during the course of the disease, indicating the physiological significance of the increase in the fibronectin receptor. This study provides the missing piece of the mechanistic puzzle concerning the actions of TGF-β. Thus, both increased synthesis and decreased degradation of matrix coupled with increases in the number of integrin receptors on glomerular cells contribute to the deposition of matrix components and the accumulation of pathologic matrix following glomerular injury.

## Crescentic glomerulonephritis

In previous studies, Coimbra *et al.* [18] demonstrated increased TGF-β production in glomeruli isolated from rabbits with anti–glomerular basement membrane crescentic glomerulonephritis. In this model, the kidney develops rapid cortical fibrosis due to induction of interstitial collagen production immediately following glomerular injury [19]. In a new report, this group measured TGF-β levels in urine from the nephritic animals [20]. TGF-β activity was found in both normal and nephritic urine and was expressed in relation to urine creatinine concentration. In the urine of nephritic animals, TGF-β activity was increased from day 2 of disease, peaked on day 7, and returned to normal by day 14. This time course paralleled TGF-β production by isolated nephritic glomeruli. When TGF-β levels for individuals animals were compared with the severity of cortical fibrosis, a significant positive correlation was found. The results suggest that urinary TGF-β activity may be a useful predictor of fibrogenesis and progression to end-stage disease.

## Puromycin aminonucleoside–induced nephrosis

A single injection of puromycin aminonucleoside into a rat results in an acute, reversible nephrotic syndrome that has been used as a model of minimal-change disease in humans. One week following induction of nephrosis, elevated levels of TGF-β were found in whole kidney tissue that remained increased for 3 weeks [21]. TGF-β is a potent chemoattractant for monocyte-macrophages, and a significant macrophage infiltration was detected in the tubulointerstitium. With activation of TGF-β, there was induction of types I and IV collagen and fibronectin expression in the interstitium along with an increase of the tissue inhibitor of metalloproteinase. These events resulted in transient matrix protein deposition in the interstitium, but when TGF-β expression declined, the histologic appearance of the tissues returned to normal. The authors suggest that interstitial macrophages may be a major source of TGF-β production in this acute model of nephrosis.

Hyperlipidemia in nephrotic syndrome is alleged to be a progression factor for human glomerular disease. In an interesting report, rats with puromycin aminonucleoside–induced nephrosis were placed on normal or augmented-cholesterol diets [22]. Cholesterol feeding was found to increase glomerular TGF-β expression in the nephrotic rats as well as in normal rats. Increased TGF-β expression also correlated with increased expression of fibronectin, a major component of the mesangial matrix. Again, infiltrating macrophages were thought to be the cells responsible for the TGF-β production. These results suggest a molecular mechanism involving TGF-β by which hyperlipidemia might accelerate the development of glomerulosclerosis.

## Diabetic nephropathy

The central pathologic feature of diabetic nephropathy is expansion of the mesangial matrix. In glomeruli of rats made diabetic with streptozotocin, a slow progressive increase in expression of TGF-β mRNA and protein was found [23••]. Matrix proteins known to be induced by TGF-β were deposited within the glomeruli, indicating that TGF-β was biologically active in inducing matrix deposition in the diabetic animals. Administration of insulin reduced TGF-β levels but not to normal levels. These results suggest that hyperglycemia in some way is linked to the elevated TGF-β expression. This is the first report directly implicating a cytokine in the pathogenesis of diabetic nephropathy.

## Toxicity studies

Investigators at Genentech Inc. (San Francisco, CA) performed toxicity studies with recombinant TGF-β in rats [24]. They found that glomerulosclerosis developed in 14 days in 30% of rats injected daily with 100 μg of TGF-β and in 80% injected with 1000 μg. Liver fibrosis occurred in 50% and 100%, respectively, of the same rats. Thus, the dramatic fibrogenic potential of TGF-β already described and proven in actual models of disease was confirmed by these pharmacologic experiments.

## Human glomerulonephritis

Expression of TGF-β in normal and glomerulonephritic kidneys was examined by immunohistochemistry and *in situ* hybridization [25•]. The results are exactly what would be predicted from the animal models previously described. Glomerular staining of TGF-β was strongly

positive in IgA nephropathy and other forms of mesangial proliferative glomerulonephritis. Furthermore, the intensity of TGF-β staining highly correlated with the degree of mesangial matrix expansion. The cells expressing TGF-β were found to be resident glomerular cells rather than infiltrating macrophages.

## Human diabetic nephropathy

Kidney tissue from patients with diabetic glomerulosclerosis was examined for TGF-β protein and an isoform of fibronectin known to be induced by TGF-β [23••]. All of the glomeruli from the patients with diabetic glomerulosclerosis were strongly positive for TGF-β and the fibronectin isoform. Control glomeruli from normal individuals and others with minimal-change disease or thin basement membrane disease were negative.

## Transforming growth factor-β antagonists as drugs

Antibodies to TGF-β have been successfully employed in the kidney and skin to prevent matrix deposition and scarring [1•]. Recently, the proteoglycan decorin was shown to bind TGF-β and neutralize its action [26]. This finding suggested that decorin might be a natural regulator of TGF-β. Decorin was tested in the model of acute mesangial proliferative glomerulonephritis [27••]. Injections of decorin strongly reduced matrix protein deposition in the nephritic glomeruli, suppressed proteinuria, and ameliorated histologic manifestations of the disease. Suppressing TGF-β did not interfere with normal glomerular healing or have any other deleterious effects. Decorin may be particularly suitable for treatment of kidney disease because of a propensity to accumulate in the kidney following intravenous injection. Also, because decorin is a natural human compound, it offers hope as a treatment for human fibrotic diseases caused by TGF-β.

## Conclusions

The findings in cell culture, experimental models, and human disease strongly implicate TGF-β as the major cytokine responsible for extracellular matrix deposition and scarring in glomerular disease. TGF-β antagonists, such as decorin, may eventually be clinically useful in glomerular diseases associated with the overproduction of TGF-β.

## Acknowledgments

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# Cyclosporin inhibits intercellular adhesion molecule-1 expression and reduces mast cell numbers in the asebia mouse model of chronic skin inflammation.

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The asebia mouse represents a spontaneous mutation in BALB/c mice leading to hyperplasia of the epidermis and chronic inflammatory dermal changes including enhanced cellularity, oedema and elevated mast cell numbers. We demonstrated that asebia mice have constitutively elevated intercellular adhesion molecule-1 (ICAM-1) mRNA expression, which is not detectable in the wild type, and that dermal mast cell numbers were 3.1-fold higher than the wild type ($P < 0.001$). We utilized this model to explore the anti-inflammatory effects of cyclosporin A (CsA). After 3 weeks subcutaneous injection with 5 or 10 mg/kg CsA the expression of ICAM-1 mRNA was determined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and was found to be decreased 2.7-fold ($P < 0.001$) and three-fold ($P < 0.001$) relative to controls for 5 and 10 mg/kg treatments, respectively. Dermal mast cell counts were dose-dependently decreased by CsA. Mast cells, visualized by toluidine blue staining, decreased 4.5-fold with 10 mg/kg CsA ($P < 0.001$) bringing them down to numbers typical of the wild type. CsA also appeared to stabilize mast cell histamine content. Histological examination of haematoxylin-eosin-stained sections revealed that CsA treatment restored the wild-type skin phenotype decreasing epidermal hyperplasia, dermal cellularity and oedema. Thus, CsA exhibits a wide range of anti-inflammatory effects including reduction of ICAM-1 expression and mast cell numbers, and may be useful in

the treatment of mast cell-mediated dermatoses.

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# Cyclosporine A regulates T cell-epithelial cell adhesion by altering LFA-1 and ICAM-1 expression

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**Cyclosporine A regulates T cell-epithelial cell adhesion by altering LFA-1 and ICAM-1 expression.** In contrast to the well characterized suppressive effect of cyclosporine A (CsA) on IL-2 gene transcription in T cells, other immunosuppressive effects of CsA have received less attention. We have examined the effect of CsA on the expression of the $\beta_2$ integrin, LFA-1, and its counter receptor, ICAM-1, on a renal Ag-specific murine T cell clone and Ag-expressing renal tubular epithelial cells. We have found that CsA has a concentration dependent effect on the expression of both ICAM-1 mRNA and gene product on renal tubular cells. At 0.1 μg/ml, CsA exhibits a costimulatory effect, with TNFα, on ICAM-1 expression. CsA at 1 to 5 μg/ml exhibits concentration dependent inhibition of ICAM-1 cell surface expression by the tubular cells. Although CsA does not inhibit ICAM-1 on T cells, it does inhibit surface expression of LFA-1. The concentration dependent effects of CsA on ICAM-1 expression correlate well with ICAM-1 dependent T cell adhesion to TNFα stimulated tubular epithelial cells. TGF-β1 has similar effects on ICAM-1 and LFA-1 expression as high dose CsA, but the CsA effects are not mediated through induced TGF-β1 expression. Our studies support the conclusion that CsA may bidirectionally alter ICAM-1 dependent cellular adhesive interactions. The inhibition of cytokine stimulated ICAM-1 expression at higher CsA concentrations would contribute to the overall immunosuppressive effect of the drug.

Cyclosporine A (CsA) is known to exert an immunosuppressive effect by inhibiting T cell activation and function [1]. It is widely used to prevent organ transplant rejection [2] and increasingly to treat autoimmune diseases [3]. Most studies examining the immunosuppressive effect of CsA on T lymphocytes have focused on its profound inhibitory effect on IL-2 gene transcription and the mechanisms underlying that effect. The effect of CsA on other costimulatory molecules participating in T cell activation has not been widely investigated.

Leukocyte-function associated antigen-1 (LFA-1) and its counter-receptor intercellular adhesion molecule-1 (ICAM-1) can provide such a co-stimulatory pair of molecules [4, 5]. Typically, expression of ICAM-1 on target cells is significantly augmented by inflammatory cytokines such as γIFN and TNFα [6]. LFA-1 is constitutively expressed on T cells and its avidity to ICAM-1 increases with signals transduced through the engagement of the TCR [7, 8].

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In immune-mediated dermatologic disorders, CsA therapy is associated with a diminution in the disease-associated increase in ICAM-1 expression on endothelial cells [9], follicular epithelial cells [10] and keratinocytes [11, 12]. While such an effect may be in part secondary to suppressed cytokine expression, *in vitro* studies have demonstrated that CsA can inhibit Ag presentation by human and murine Langerhans cells, an effect not attributable to changes in class II MHC expression, IL-1 activity, carryover of CsA, or increased prostaglandin concentrations [13, 14].

We have previously described the characteristics of a panel of Ag-specific T cell clones which directly effect interstitial renal injury in SJL mice [15]. These T cells are specific for an Ag, 3M-1, expressed by renal tubular epithelial cells [16]. A proximal renal tubular epithelial cell line from SJL mice (MCT) synthesizes 3M-1 and can present this Ag to class II restricted, 3M-1 specific $CD4^+$ T cell clones [17] [18]. MCT cells also serve as targets for class I restricted, 3M-1 reactive $CD8^+$ effector T cells [15]. We utilized this system to examine whether CsA might additionally be immunosuppressive by altering the expression of LFA-1 and ICAM-1 on autoimmune T cells and their parenchymal target cells.

## Methods

### *Cells*

The MCT (mouse cortical tubule) cell line was derived from kidneys of normal SJL mice. Proximal tubular cells were selected by immunodissection using specific antibodies against the 3M-1 glycoprotein, a target antigen for autoimmune interstitial nephritis [16]. These cells were virally transformed with a non-replicating, non-capsid forming strain of SV40 and further characterized as previously described [17]. They were grown at 37°C/5% $CO_2$ in DMEM (JRH Biosciences, Lenexa, KS, USA) with 10% heat inactivated FCS. Cells were passaged every 48 to 72 hours by trypsinization. M52.28.1 is a $CD4^+$ T cell clone derived from SJL mice immunized with a rabbit renal tubular antigen in CFA. M52.28.1 mediates DTH responses to collagenase solubilized RTA, or purified 3M-1. Cells were propagated by weekly passage with 20 μg/ml antigen, 7.5 U/ml recombinant mouse IL-2 (Boehringer-Mannheim) and irradiated (4000 rads) syngeneic spleen cells. T cell culture medium consisted of RPMI 1640 (Whittaker Bioproducts, Inc., Walkersville, MD, USA) supplemented with glutamine, antibiotics (penicillin and streptomycin), 10% heat inactivated FCS, and $2 \times 10^{-5}$ M 2-ME. T cells were cultured at 37°C in a 5% $CO_2$ incubator.

### *Reagents*

Recombinant human TNFα was purchased from Boehringer (Mannheim, Indianapolis, IN, USA). Cyclosporine A was kindly donated by Sandoz (East Hanover, NJ, USA). CsA was dissolved in either 0.1% DMSO or 0.5% ethanol in DMEM. Final DMSO concentration in cultures was less than 0.01%, and ethanol less than 0.05%. TGF-β1 (recombinant human), neutralizing antisera to TGFβ, or its control chicken IgY were purchased from R&D systems (Minneapolis, MN, USA).

### *Northern blot analysis*

Total RNA was isolated from confluent monolayers of MCT or T cells by the single-step method of acid guanidium thiocyanate-phenol chloroform extraction [19]. Ten micrograms of total RNA were electrophoresed in a 1.2% agarose gel containing 2.2 M formaldehyde. RNA transfer to a positively charged nylon membrane (Zetabind, CUNO Laboratory Products, Meriden, CT, USA) was performed by capillary blotting, and followed by short wave UV cross-linking (UV Stratalinker, Stratagene, La Jolla, CA, USA). Prehybridization was performed at 55°C for five hours in a buffer containing 0.5 M $Na_2PO_4$, 7% SDS, 1% BSA, and 1 mM EDTA with 50 μg/ml polyadenylic acid (Boehringer Mannheim Inc.) and 50 to 100 μg/ml sheared salmon sperm DNA. The insert of K3-1.1 clone encoding murine ICAM-1 was used as a cDNA probe (obtained from the ATCC) [20]. A PCR-product (sequenced verified) derived from a published murine TGF-$\beta_1$ sequence was used as a probe for TGF-$\beta_1$. Probes were labeled by the random hexamer priming technique (Boehringer Mannheim) to high specific activity. Blots were hybridized in the aforedescribed buffer for 20 hours at 55°C, washed with 1 × SSC, 0.1% SDS for 20 minutes at 55°C and with 0.3 × SSC, 0.1% SDS at 60°C for 20 minutes. Membranes were then exposed to Kodak XAR-5 film. Blots were re-hybridized with a GAPDH probe as a housekeeping gene.

### *Cytofluorography*

Cytofluorography was performed on M52.28.1 cells treated with CsA or vehicle, or TGF-β1 and harvested from day 10 cultures. It was also performed on confluent MCT cells cultured in the presence of TNFα and various doses of CsA, or vehicle, and harvested after 48 to 72 hours. Cells were washed in PBS, and aliquots of 1 to 2 × $10^6$ cells were suspended in 50 μl PBS with 0.1% BSA (staining buffer). FITC-conjugated αLFA-1 monoclonal antibodies (PharMingen, San Diego, CA, USA) or αICAM-1 monoclonal antibodies (BE3E5, gift from A.A. Brian, La Jolla Cancer Research, La Jolla, CA, USA) were incubated with samples on ice for 20 minutes. Following a wash with staining buffer, samples stained with αICAM-1 Abs were incubated with FITC-conjugated goat anti-rat IgG for 20 minutes on ice. Cells were fixed in 500 μl 1% formaldehyde in PBS. Fluorescence was recorded on a FACScan® cytofluorograph (Becton Dickinson & Co., Mountain View, CA, USA). In each run, 10,000 live gated cells were analyzed.

### *Adhesion assays*

MCT cells were grown to confluence in 24-well plates in DMEM supplemented with 10% FCS. TNFα (100 ng/ml) and various doses of CsA (or its vehicle) were added to the media and the assay was performed 48 hours later. Media was changed prior to the assay and replaced with DMEM/10% FCS (no TNFα or CsA). M52.28.1 T cells (5 to 7 days following passage) were pulsed with 1 μCi/ml $^3$H-TdR for 16 hours. Culture medium was then replenished, T cells were washed, and 1 × $10^5$ T cells/well were added to the MCT cells. Plates were shaken briefly to bring T cells into contact with the monolayers and then incubated at 37°C. After one hour, non-adherent cells were washed by flicking the plates rapidly and adding fresh warm media. This was repeated four times. Cells were then transferred to 96 well plates, harvested and counted in quadruplicate using a β-counter. Blocking antibodies to ICAM-1 (BE2901, a gift from A.A. Brian) or control rat IgG were added to the MCT cells one hour prior to co-incubation with M52.28.1. As performed, this assay does not examine VLA-4 mediated adhesion, since VLA-4 is not expressed on this T cell clone at this time point following passage (C. Kelly and A. Deng, unpublished observations).

## Results

### *CsA modulates ICAM-1 cell-surface expression in tubular epithelial cells stimulated with TNFα*

We first evaluated the effect of CsA on the cell surface expression of ICAM-1 using cytofluorography. Following growth to confluence in DMEM and 10% FCS, MCT cells were cocultured with TNFα (100 ng/ml) and graded concentrations of CsA or vehicle for 48 hours. At the concentrations used, CsA did not alter the proliferation of MCT cells. Unstimulated MCT cells show a very low basal level of ICAM-1 cell surface expression (Fig. 1). This low basal level of expression increased significantly following TNFα treatment and was even further augmented by coculture with 0.1 μg/ml CsA (Fig. 1). This stimulatory effect of TNFα was abrogated when CsA was added at 1 to 5 μg/ml. The biphasic, dose-dependent effect of CsA depicted in Figure 1 is representative of four individual experiments. CsA had no stimulatory effect on ICAM-1 expression at 0.1 μg/ml in the absence of TNFα or when expression of tubular cell ICAM-1 was maximally stimulated by coculture with both TNFα and γIFN (data not shown). CsA at 1 to 5 μg/ml did not inhibit tubular cell ICAM-1 expression stimulated by the combination of TNFα and γIFN (data not shown). We obtained similar results to those depicted in Figure 1 if the cells were harvested following only 24 hours of exposure to TNFα and CsA or if the cells were preincubated with TNFα for 24 hours prior to CsA administration (data not shown).

### *CsA modulates steady-state mRNA levels of ICAM-1 in tubular epithelial cells*

We next examined ICAM-1 transcript levels in the same experimental groups by Northern blot analysis. The blot was hybridized to a full-length cDNA probe encoding murine ICAM-1 [20]. This probe identified a 3.2 kb band as the size of the ICAM-1 mRNA expressed by MCT cells (Fig. 2). This ICAM-1 mRNA was not detected in unstimulated MCT cells but was up-regulated by TNFα and further augmented in the presence of both TNFα and 0.1 μg/ml CsA. The stimulatory effect of TNFα was significantly

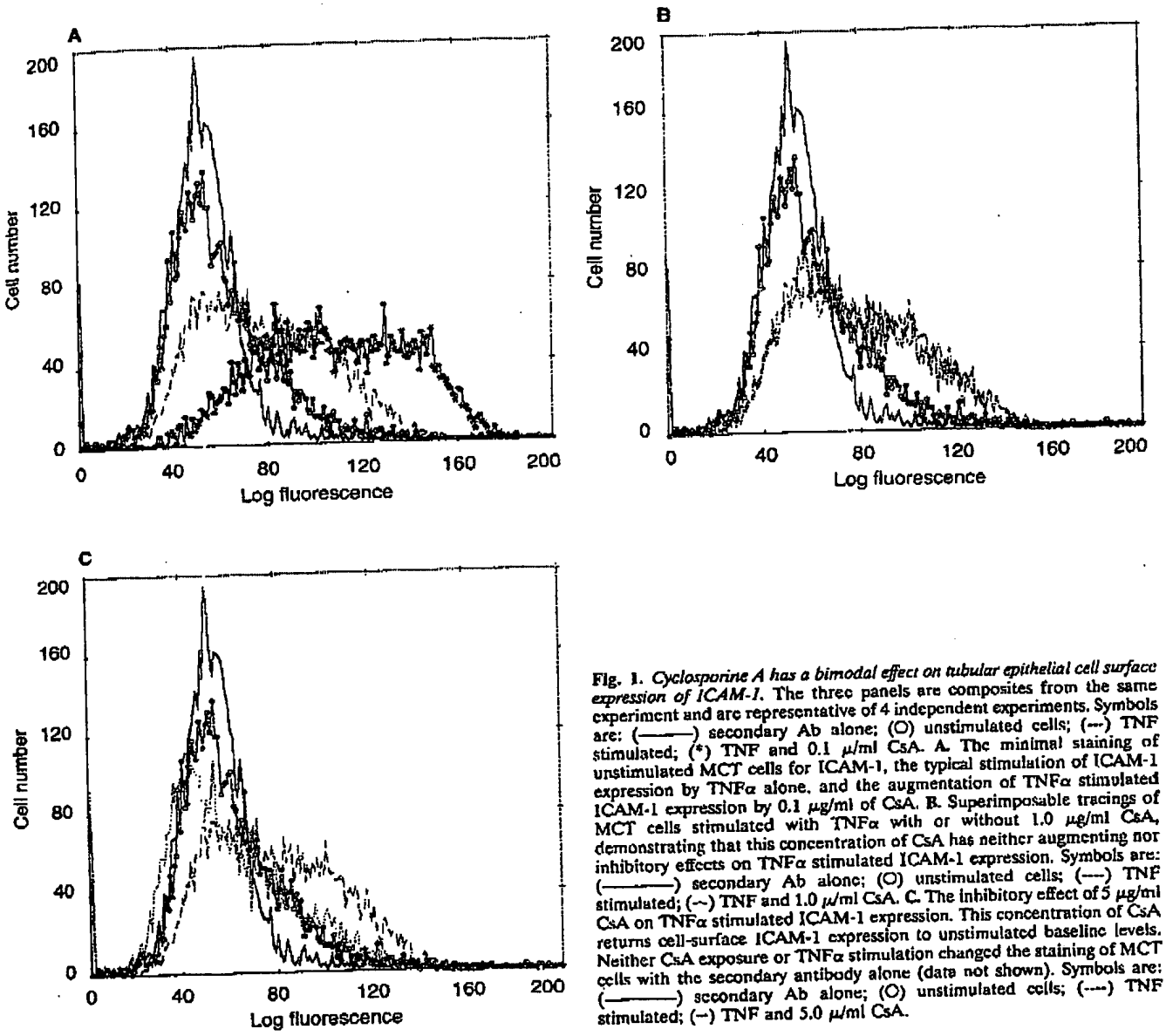


**Fig. 1.** *Cyclosporine A has a bimodal effect on tubular epithelial cell surface expression of ICAM-1.* The three panels are composites from the same experiment and are representative of 4 independent experiments. Symbols are: (———) secondary Ab alone; (O) unstimulated cells; (---) TNF stimulated; (*) TNF and 0.1 μ/ml CsA. A. The minimal staining of unstimulated MCT cells for ICAM-1, the typical stimulation of ICAM-1 expression by TNFα alone, and the augmentation of TNFα stimulated ICAM-1 expression by 0.1 μg/ml of CsA. B. Superimposable tracings of MCT cells stimulated with TNFα with or without 1.0 μg/ml CsA, demonstrating that this concentration of CsA has neither augmenting nor inhibitory effects on TNFα stimulated ICAM-1 expression. Symbols are: (———) secondary Ab alone; (O) unstimulated cells; (---) TNF stimulated; (--) TNF and 1.0 μ/ml CsA. C. The inhibitory effect of 5 μg/ml CsA on TNFα stimulated ICAM-1 expression. This concentration of CsA returns cell-surface ICAM-1 expression to unstimulated baseline levels. Neither CsA exposure or TNFα stimulation changed the staining of MCT cells with the secondary antibody alone (data not shown). Symbols are: (———) secondary Ab alone; (O) unstimulated cells; (---) TNF stimulated; (--) TNF and 5.0 μ/ml CsA.

inhibited by CsA at 1 μg/ml and entirely abrogated by 5 μg/ml. Densitometric analysis of the ICAM-1 and GAPDH bands was performed in order to determine relative ICAM-1 mRNA levels, given the disparate loading of lane c. If the TNFα stimulated ICAM-1 (lane b) was expressed as 100 arbitrary units (AUs), the normalized lane c was 200 AUs, and normalized lane d, 50 AUs. These relative steady-state mRNA levels were consistent with the modulation of stimulated ICAM-1 cell surface expression by CsA.

### *CsA inhibition of ICAM-1 cell surface expression in TNFα stimulated MCT cells is independent of TGFβ*

Recent studies have indicated that CsA can stimulate the expression of TGF-β1 [21]. We examined whether this might be a mechanism underlying the inhibitory effect of CsA depicted in Figure 1. Figure 3 demonstrates that MCT cells cocultured with CsA displayed a concentration dependent increase (when normalized for GAPDH) in the 2.5 kb TGF-β1 transcript. Figure 4 shows that exogenous $TGF\beta_1$ can inhibit TNFα stimulated ICAM-1 expression on MCT cells, much like the effect of higher concentrations of CsA. Since CsA and TGFβ had similar effects on ICAM-1 expression, we examined whether the inhibitory effect of 5 μg/ml CsA was mediated via induced TGFβ1. MCT cells were co-stimulated with TNFα and CsA in the presence of neutralizing antisera to $TGF\beta_1$ (25 μg/ml). The results shown in Figure 5 demonstrate that the induction of ICAM-1 cell surface expression by TNFα was abrogated by the co-administration of CsA (5

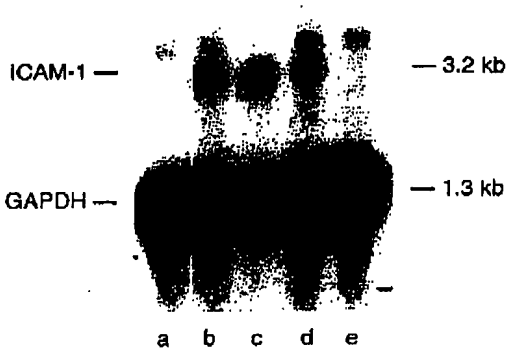


Fig. 2. *CsA has a bimodal effect on ICAM-1 mRNA levels in MCT cells.* Lane a demonstrates the absence of ICAM-1 mRNA in confluent MCT cells treated with the CsA diluent alone. Following stimulation with TNFα (lane b) and diluent a 3.2 kb mRNA species is induced. This same hybridizing band is superinduced in the presence of TNFα and 0.1 μg/ml CsA (lane c). Steady state mRNA ICAM-1 levels are partially inhibited by 1.0 μg/ml CsA (lane d) and completely inhibited by 5 μg/ml CsA (lane e).

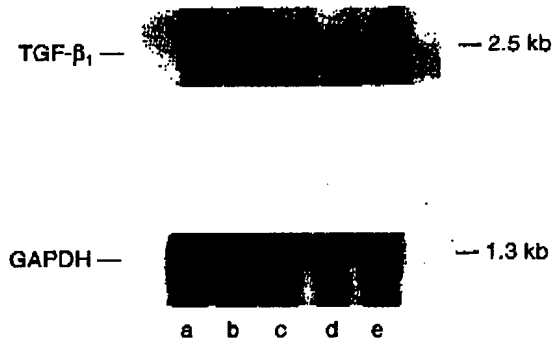


Fig. 3. *CsA induces TGFβ mRNA in MCT cells.* MCT cells were incubated for 48 hours in the presence of varying concentrations of CsA or vehicle alone (lane a). The final CsA concentrations were 100 ng/ml (lane b), 1.0 μg/ml (lane c), 3.0 μg/ml (lane d), or 5 μg/ml (lane e).

μg/ml). This inhibitory effect was not reversed by the presence of neutralizing antisera to TGFβ$_1$ or the control antisera. This experiment was representative of three. No effect was seen even when the neutralizing antisera was used at 50 μg/ml. As a control for the efficacy of the neutralizing antisera, preliminary studies demonstrated that 25 μg/ml of the neutralizing antisera completely blocked the inhibitory effect on ICAM-1 expression of 5 ng/ml exogenous TGFβ1. This concentration of antisera neutralized the inhibitory effect of 10 ng/ml TGFβ1 by approximately 75 to 80%, while 50 μg/ml neutralizing antisera completely inhibited the effect of 10 ng/ml TGFβ1 (data not shown). We conclude that, despite the similar inhibitory effect of CsA and TGFβ on ICAM-1 expression in TNFα treated MCT cells, CsA exerts its effect by a mechanism independent of TGFβ.

### *CsA alters the adhesion of M52.28.1 to TNFα-treated MCT cells*

We conducted a number of functional studies examining whether this effect of CsA on ICAM-1 in MCT cells altered the interaction between a 3M-1 reactive T cell clone and the Ag-expressing MCT cells. Adhesion studies were performed as described in the **Methods**. We found that there is over a twofold increase in M52.28.1 adherence to TNFα-treated MCT cells as compared to unstimulated cells (Fig. 6). This increase is due to up-regulation of ICAM-1, as it is inhibited by anti-ICAM-1 blocking antibodies but not by the control rat IgG. Co-administration of TNFα and low dose CsA (0.1 μg/ml) further augments the adhesion of M52.28.1 to MCT cells, again in an ICAM-1-dependent mechanism. CsA in higher dose (5 μg/ml) had a marked inhibitory effect on the adhesion of M52.28.1 to TNFα-treated MCT cells. (It should be noted that the design of this experiment excluded the possibility of VLA-4/VCAM interactions since VLA-4 was not expressed on the T cells at this stage following passage.) We conclude that CsA alters the adhesion of a nephritogenic T cell clone to tubular epithelial cells by modulating the expression of ICAM-1 on the target cell.

### *CsA down-regulates LFA-1 expression in a CD4$^+$ effector T cell clone*

M52.28.1 is a CD4$^+$ DTH-reactive T cell clone. It will result in interstitial nephritis following adoptive transfer into syngeneic recipients [22]. Since CsA was found to down-regulate ICAM-1 expression on target cells, we examined its effect on M52.28.1 LFA-1 expression. M52.28.1 was cultured with 10 to 1000 ng/ml of CsA beginning on the day of passage and followed by repeated administration every 48 hours. Control cells were treated with an equal volume of the vehicle, DMSO. M52.28.1 cells were harvested on day 7 following treatment with CsA, and tagged with FITC-conjugated αLFA-1 mAbs. CsA exhibited a concentration dependent inhibitory effect on LFA-1 cell surface expression in M52.28.1 cells with 53% inhibition with the highest dose tested (1 μg/ml) (Fig. 7A). This concentration of CsA also inhibited steady-state mRNA levels of both the LFA-1 α and β$_2$ chains (data not shown). In parallel studies, we additionally examined the expression of ICAM-1 on M52.28.1 and the effect of CsA on this expression. Unlike the MCT cells, CsA did not modulate (in either direction) the expression of ICAM-1 on M52.28.1 cells (data not shown).

The inhibitory effect of CsA on LFA-1 expression in M52.28.1 was independent of TGFβ. With a rationale similar to that expressed above, M52.28.1 cells were cultured in the presence of various concentrations of exogenous TGFβ1 (0.1 to 5.0 ng/ml) and LFA-1 expression on treated cells examined on day 7. Figure 7B demonstrates that TGFβ down-regulated LFA-1 cell surface expression in M52.28.1 cells. Fifty-four percent inhibition was noted with the highest dose tested (mean channel of 7.4 vs. 13.6). Since both CsA and TGFβ1 had a similar inhibitory effect on LFA-1 cell surface expression in M52.28 cells, we examined whether this effect was interrelated. Cells were co-incubated with CsA (1 μg/ml) and neutralizing antisera to TGFβ (50 μg/ml). The results depicted in Figure 7C demonstrate that the inhibitory effect of CsA on LFA-1 expression could not be reversed by neutralizing antibodies to TGFβ or its control antisera. This concentration of TGFβ1 antisera fully blocked the inhibitory effect of 5.0 ng/ml exogenous TGFβ1 on T cell LFA-1 expression (data not shown). We conclude that CsA down-regulates LFA-1 expression by a mechanism independent of induction of TGF-β1.

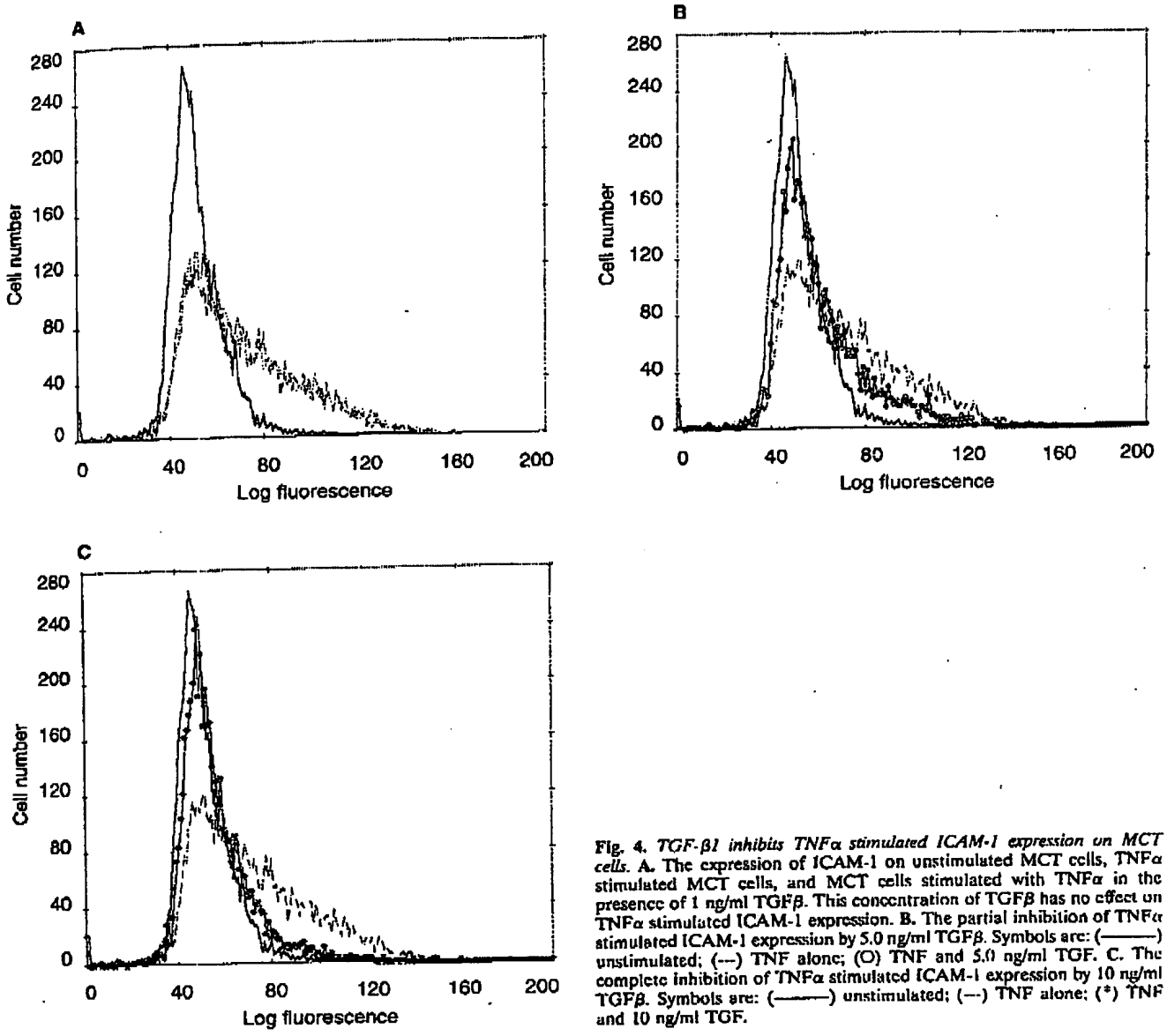


Fig. 4. *TGF-β1 inhibits TNFα stimulated ICAM-1 expression on MCT cells.* A. The expression of ICAM-1 on unstimulated MCT cells, TNFα stimulated MCT cells, and MCT cells stimulated with TNFα in the presence of 1 ng/ml TGFβ. This concentration of TGFβ has no effect on TNFα stimulated ICAM-1 expression. B. The partial inhibition of TNFα stimulated ICAM-1 expression by 5.0 ng/ml TGFβ. Symbols are: (———) unstimulated; (---) TNF alone; (O) TNF and 5.0 ng/ml TGF. C. The complete inhibition of TNFα stimulated ICAM-1 expression by 10 ng/ml TGFβ. Symbols are: (———) unstimulated; (---) TNF alone; (*) TNF and 10 ng/ml TGF.

## Discussion

The studies presented here demonstrate that CsA modulates ICAM-1 cell-surface expression on renal tubular epithelial cells treated with TNFα. This modulatory effect correlates well with the steady-state mRNA levels. CsA exhibits a differential effect on ICAM-1, depending on the concentration and cell type used. Low concentrations of CsA (0.1 μg/ml) have a costimulatory effect when administered with TNFα, whereas the higher concentrations are inhibitory. CsA does not influence ICAM-1 expression in M52.28.1 cells. CsA down-regulates LFA-1 cell surface expression on M52.28.1 cells, in a dose-dependent fashion. The effect of CsA on ICAM-1 in MCT cells and LFA-1 in M52.28.1 is independent of TGFβ. The effect of CsA on ICAM-1 expression in antigen-expressing epithelial cells (MCT) is functionally significant in that it modulates their adherence to an antigen-reactive T cell clone (M52.28.1).

Antigen-independent contact between T cells and target cells, mediated by adhesion molecules including LFA-1/ICAM-1, is an initiating event in T cell recognition [23]. The importance of this receptor-ligand pair was demonstrated by the capacity of monoclonal antibodies to inhibit T cell adhesion to target cells and subsequently cytolysis [24, 25]. The cell surface expression of ICAM-1 frequently parallels class II MHC expression, and is up-regulated in various disease states. It is induced on human renal tubular cells during allograft rejection [26] and in a murine model of lupus nephritis [27]. Such induction appears to be the

# Acute Cyclosporine-Induced Nephrotoxicity in Renal Transplant Recipients: The Role of the Transplanted Kidney[1]

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## ABSTRACT

Cyclosporine A causes an acute reduction in GFR. The interindividual variable reduction in GFR is most likely the result of arteriolar vasoconstriction. Vasoconstriction is attributable either to a local effect of cyclosporine on renal blood vessels (intrinsic mechanism) or to a systemic effect of cyclosporine on circulating and/or neuronal factors (extrinsic mechanism). The aim of the investigation presented here was to establish whether intrinsic or extrinsic mechanisms account for the interindividual differences in the susceptibility to acute cyclosporine-induced nephrotoxicity. For that purpose, this study took advantage of the clinical transplant situation in which two (intrinsically identical) kidneys from a cadaveric donor are transplanted into two (extrinsically) different subjects. The preexisting regular daily cyclosporine doses were raised by 25% for 2 wk and by 50% for another 2 wk in 16 patients with stable renal graft function, representing eight pairs of patients, each of whom had received kidneys from the same donor. In these patients, a mean (± SD) maximum cyclosporine-induced increase in serum creatinine concentration of 13 ± 11% ($P < 0.001$) and in serum BUN of 27 ± 33% ($P < 0.01$), together with a decline in the fractional uric acid excretion of 51 ± 89% ($P < 0.02$) were observed. The percentage change in serum creatinine concentrations after increased dosing of cyclosporine paralleled within the subjects receiving their kidneys from the same donor, *i.e.*, when one recipient experienced a large percentage of change after increases of cyclosporine dosing, the corresponding recipient of a kidney from the same donor had a change of the same magnitude. Seven of eight pairs showed a consistent response with respect to a clinically significant increase in serum creatinine concentration of >15%, with a consistent response purely by chance being <5%. Thus, the transplanted kidney itself rather than the recipient determines the susceptibility to acute cyclosporine-induced nephrotoxicity.

Key Words: *Cyclosporine, toxicity, renal failure, transplantation, humans*

Dose- and time-dependent renal dysfunction (1–3) is the major side effect observed during immunosuppression with cyclosporine. Acute cyclosporine-induced renal dysfunction is nonprogressive, dose-dependent, and reversed by dose reduction or discontinuation (4–6).

The precise pathomechanism underlying cyclosporine's nephrotoxicity remains unclear. Morphologic and functional studies in animals (7–9) and man (10–12) have suggested that increased arteriolar resistance with a predominant afferent arteriolar vasoconstriction is the major mechanism accounting for the acute reduction in GFR when cyclosporine is given. Vasoconstriction might be a result of a local effect of cyclosporine on renal blood vessels or of a systemic effect of cyclosporine on circulating and/or neuronal factors. Thus, the large interindividual differences in the decline of the GFR after cyclosporine dosing (10,11) can be explained either by interindividual differences of the (intrinsic) susceptibility of the kidney itself or by a variable (extrinsic) response of the rest of the body. Donor age (13–15), prolonged warm ischemia time (16,17), and preexisting ischemic injury to cadaver kidneys (18,19) have all been reported as potential risk factors for graft loss. However, nothing is known, to the best of our knowledge, about a potential role of the kidney donor in the individual recipients' susceptibility to cyclosporine-induced nephrotoxicity.

The aim of the investigation presented here was, therefore, to establish whether intrinsic or extrinsic mechanisms account for the interindividual differences in the susceptibility to acute cyclosporine-induced nephrotoxicity. For that reason, we took advantage of the clinical transplant situation in which two (intrinsically identical) kidneys from a cadaveric donor are transplanted into two (extrinsically) different subjects. We hypothesized that a synchronous decline in GFR in pairs of stable kidney transplant recipients by temporarily increasing cyclosporine doses indicates intrinsic organ (or donor) factors, whereas a differential modulation in GFR indicates extrinsic (or recipient) factors.

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## METHODS

### Patients

Sixteen patients (nine women, seven men; mean age, 47 years; age range, 28 to 66 yr) with stable functioning renal grafts were studied. These patients represented eight pairs of kidney transplant recipients, each pair with kidneys from the same donor. All recipients were transplanted at least 2 yr before the study. They were on stable long-term immunosuppression with either cyclosporine alone or in combination with prednisone and/or azathioprine (Table 1). All patients had stable renal function and cyclosporine regimen documented by cyclosporine whole blood concentrations, serum creatinine values, and creatinine clearance measurements over the last 2 months before the study.

Patients with diabetes, nonsteroidal anti-inflammatory drug intake and/or uncontrolled hypertension (as defined by a diastolic blood pressure above 105 mm Hg) were excluded. The study was approved by the ethical committee of the University of Berne, and all patients gave their informed consent to undergo the following study protocol.

### Study Protocol

The patients remained ambulatory. Cyclosporine was administered orally as a single daily dose, and pre-existing concurrent medications were continued throughout the study. After evaluation of baseline clinical and laboratory parameters (see below), the daily dosage of cyclosporine was raised by 25% for 2 wk. After this period, an additional increase of 25% of the daily dosage of cyclosporine was prescribed for another 2 wk, and thereafter the daily dosage of cyclosporine was reduced back to baseline dosage.

Throughout the study, all patients were seen weekly and the following clinical and laboratory parameters were collected: body weight, blood pressure, heart rate, and, possibly, cyclosporine-induced clinical side effects, such as tremor. Weekly laboratory analyses included cyclosporine whole blood concentrations (trough levels 24 h after the last dose), BUN, serum creatinine, uric acid, potassium, and magnesium levels, as well as total serum bilirubin concentrations. Urine creatinine and uric acid were measured weekly from two 24-h urine collections, and creatinine and uric acid clearances as well as the fractional excretion of uric acid were calculated.

Serum and urine samples were analyzed by standard biochemical assays. Whole blood cyclosporine concentrations were determined by using a specific fluorescence polarization immunoassay (Abbott TDx; Abbott Laboratories, North Chicago, IL) (20).

Criteria for immediate suspension from the study were a diastolic blood pressure above 105 mm Hg, an increase of serum creatinine levels by more than 50% as compared with baseline values, and/or a rise in serum potassium level above 5.5 mmol/L.

TABLE 1. Patient characteristics[a]

| | |
|---|---|
| Age | 47 ± 11 |
| Body Weight (kg) | 68 ± 17 |
| Height (cm) | 165 ± 9 |
| Blood Pressure (mm Hg) | 141/90 ± 18/11 |
| Heart Rate/min | 70 ± 10 |
| Immunosuppressive Therapy | |
| Cyclosporine (mg/day) | 273 ± 136 |
| Prednisone (mg/day) | 6.2 ± 3.8 |
| Azathioprine (mg/day) | 40 ± 54 |

[a] All values given as mean ± SD.

### Statistical Analysis

Patients were grouped according to their individual response to the raised cyclosporine doses. An increase of 15% or more of the serum creatinine level during any routine control of a transplanted patient is considered a relevant clinical finding at our division. This same limit was used for the current investigation, and all patients that showed serum creatinine level increase of 15% or more in at least one of the serum creatinine measurements during the study were grouped as "responders." Independent *t* tests were used for comparisons between responders and nonresponders.

## RESULTS

The profiled increase of the daily cyclosporine dose by 25% for 2 wk and 50% for another 2 wk resulted in an increase of the daily cyclosporine dose from 271 ± 137 mg/day (mean ± SD) to 341 ± 171 mg/day and to 406 ± 207 mg/day, respectively (Table 2). The corresponding whole blood cyclosporine levels increased from 117 ± 26 to 160 ± 53 and 188 ± 42 ng/mL, respectively. All patients tolerated the increase of the daily cyclosporine dose, and all were able to finish the study protocol.

The mean of the observed individual maximum increases of serum creatinine level during the 4-wk observation period was 13 ± 11% ($P < 0.001$). This increase was accompanied by a 27 ± 33% increase in BUN ($P < 0.01$) and a 51 ± 89% decrease in the fractional excretion of uric acid ($P < 0.05$) (Table 2). Creatinine clearance and serum levels of uric acid, potassium, and magnesium showed no consistent changes.

The patients were grouped according to their individual response to the raised cyclosporine doses. An increase by 15% or more in at least one of the serum creatinine measurements was considered to be of clinical relevance. Nine patients increased their serum creatinine to levels above 15% (responders), whereas in seven patients serum creatinine levels consistently stayed below 15% during the 4 wk of the higher cyclosporine dosage (nonresponders). Responders and nonresponders not only differed with respect to their maximum increase of serum creatinine concentrations (20 ± 3 *versus* 3 ± 7%, $P < 0.001$) (Table 2) but also with respect to their mean changes in creatininine clearance (−11 ± 6 *versus* 2 ± 6%, $P < 0.01$) as well as to changes in their maximum BUN concentrations (44 ± 31 *versus* 4 ± 19%, $P < 0.01$) (Table 2).

A higher percentage increase in cyclosporine blood concentrations was noted for responders at the time of maximum serum creatinine measurements (71 ± 27 *versus* 40 ± 22%, $P < 0.05$), but the mean increase of cyclosporine blood levels, obtained by considering all of the concentrations after the increase of cyclosporine A dosing, was not significantly different between the two groups (56 ± 20 *versus* 39 ± 19%). Maximum

TABLE 2. Laboratory values at baseline and at time of maximum increase of serum creatinine concentration

| Parameter | All Patients | | Responders[a] | | Nonresponders | |
|---|---|---|---|---|---|---|
| | Baseline | Maximum[b] | Baseline[c] | Maximum[b] | Baseline[c] | Maximum[b] |
| Cyclosporine, Whole Blood Concentrations (ng/mL) | 117 ± 26 | 182 ± 48[d] | 108 ± 23 | 182 ± 46[d] | 129 ± 25 | 181 ± 53[d] |
| Serum Levels | | | | | | |
| Creatinine (μmol/L) | 126 ± 29 | 142 ± 32[d] | 124 ± 31 | 149 ± 35[d] | 129 ± 30 | 133 ± 28 |
| BUN (mmol/L) | 9.9 ± 3.2 | 12.2 ± 3.9[e] | 9.4 ± 3.3 | 13.2 ± 4.5[d] | 10.6 ± 3.1 | 10.8 ± 2.4 |
| Potassium (mmol/L) | 4.3 ± 0.7 | 4.6 ± 0.8 | 4.2 ± 0.7 | 4.6 ± 1.0 | 4.6 ± 0.5 | 4.6 ± 0.6 |
| Uric Acid (μmol/L) | 398 ± 150 | 436 ± 147 | 394 ± 161 | 459 ± 180 | 404 ± 147 | 406 ± 95 |
| Magnesium (mmol/L) | 0.76 ± 0.09 | 0.78 ± 0.14 | 0.78 ± 0.09 | 0.83 ± 0.13 | 0.74 ± 0.09 | 0.71 ± 0.13 |
| Creatinine Clearance (mL/min) | 64 ± 31 | 61 ± 30 | 73 ± 38 | 61 ± 24 | 53 ± 14 | 62 ± 37 |
| Fractional Uric Acid Excretion (%) | 11.2 ± 9.3 | 7.9 ± 6.3[f] | 12.4 ± 11.4 | 8.1 ± 8.2 | 9.5 ± 5.3 | 7.7 ± 3.1 |

[a] Individual maximum increase of serum creatinine ≥ 15%.
[b] At maximum increase of serum creatinine levels.
[c] No significant differences between responders and nonresponders were detected for any of the baseline parameters.
[d] $P < 0.001$ compared with baseline parameters.
[e] $P < 0.01$ compared with baseline parameters.
[f] $P < 0.05$ compared with baseline parameters.

serum creatinine concentrations were measured 3.6 ± 1.2 wk after the increase of the cyclosporine dose in responders and 3.0 ± 1.1 wk in nonresponders, respectively.

The 16 patients were grouped into eight pairs, each pair with grafts from the same donor. Pairs with both patients being either responders or nonresponders were defined to exhibit a consistent response to the increase in cyclosporine dosage. Such a consistent response was observed in seven of the eight patient pairs (four responder pairs and three nonresponder pairs) (Figure 1), with the probability of observing seven or more of eight pairs with consistent response purely by chance being less than 5% (9/256).

Patient Pair 5 showed no consistent response to the increase in cyclosporine dosage. Both patients of this pair compared well with respect to all of the investigated baseline parameters. Nevertheless, the one patient that showed no response to the increase in cyclosporine dosage had a stable renal function, but rather large fluctuations in serum creatinine levels of between 155 and 175 μmol/L for months before the study. It is possible that in this patient, the measured baseline serum creatinine of 176 μmol/L was too high, blunting a possible response to the increase in cyclosporine dosage.

No differences between responders or nonresponders were found with respect to baseline laboratory parameters (Table 2), time since transplantation, patient or donor age, patient or donor sex, cold or warm ischemia time, or the current use of the following antihypertensive drugs: calcium channel blockers, angiotensin-converting enzyme inhibitors, betablockers, and/or diuretics (Table 3).

## DISCUSSION

Seven of the eight patient pairs with kidneys from the same donor showed consistent responses during the elevated cyclosporine doses with respect to their maximum increase in serum creatinine and BUN

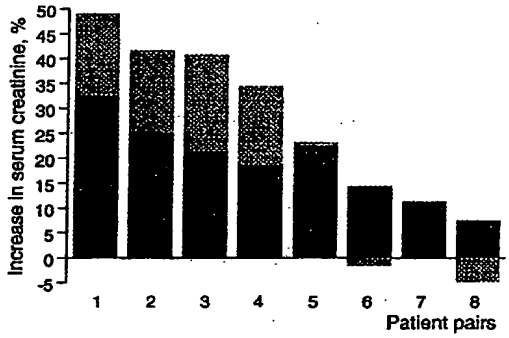


Figure 1. Maximum observed percentage increase in serum creatinine in eight patient pairs, each with grafts from the same donor. Each stacked bar combines the observed changes in serum creatinine level of one patient pair. Visual inspection reveals that the percentage change in serum creatinine levels paralleled within the subjects receiving their kidneys from the same donor, *i.e.*, when one recipient experienced a large percentage change in serum creatinine after increases of cyclosporine A dosing, the corresponding recipient of a kidney from the same donor had a change of the same magnitude. All but one pair (Patient Pair 5) showed consistent creatinine changes in response to the increased cyclosporine doses. One patient of Patient Pair 6 showed a 0% change in serum creatinine value.

TABLE 3. Comparison of parameters in responders and nonresponders[a]

| | Responders ($N$ = 9) | Non-responders ($N$ = 7) |
|---|---|---|
| Patient | | |
| Age[b] | 48 ± 12 | 45 ± 9 |
| Gender (f/m) | 4/5 | 5/2 |
| Donor | | |
| Age[b] | 28 ± 6 | 28 ± 8 |
| Gender (f/m) | 2/7 | 0/7 |
| Time Since Transplantation (yr)[b] | 4.3 ± 1.7 | 5.9 ± 2.2 |
| Cold Ischemia Time (hr)[b] | 18 ± 8 | 16 ± 7 |
| Concomitant Therapy, Number of Patients on Treatment | | |
| Prednisone | 8 | 4 |
| Azathioprine | 3 | 2 |
| Calcium channel blockers | 3 | 4 |
| Betablockers | 6 | 5 |
| ACEI[c] | 2 | 3 |
| Diuretics | 3 | 2 |

[a] No significant differences between responders and nonresponders were detected for any of the parameters.
[b] Values are given as mean ± SD.
[c] ACEI, angiotensin-converting enzyme inhibitors.

levels. The probability of observing such consistent responses in seven or more of eight pairs purely by chance is less than 5% (9 of 256), indicating that the transplanted kidney rather than the recipient accounts for the susceptibility to acute cyclosporine-induced nephrotoxicity. The profiled rise in the cyclosporine dosage during the study was paralleled by corresponding changes in whole blood cyclosporine concentrations. Although kinetic studies suggest that blood concentrations of cyclosporine are of value within certain limits for therapeutic dose finding, measuring cyclosporine blood levels proved useful in monitoring patients compliance, the most important variable of drug efficacy in outpatients (21).

Analysis of all patients as one group revealed a discrete increase of serum creatinine concentrations, whereas creatinine clearance values remained unaltered. These findings might be explained by an augmented tubular secretion of creatinine during the higher cyclosporine doses, with the possible consequence of overestimating the true GFR from creatinine clearance values in cyclosporine-treated patients (22,23). Alternatively, variations in the 24-h urine collections might obscure significant changes in creatinine clearance after increasing cyclosporine A dosage. The higher values of the coefficients of variation for creatinine clearance than for those of plasma concentrations of creatinine (Table 2) are in line with the latter hypothesis.

The observation of a more pronounced increase in BUN levels as compared with serum creatinine concentrations, as well as the decrease in the fractional excretion of uric acid, confirm previous reports that cyclosporine-induced intrarenal vasoconstriction causes a state of prerenal azotemia. This state results in an increase of the tubular uptake of urea and uric acid, which has been suggested to be a physiological response to the reduction of glomerular filtration pressure and which is completely reversible after drug reduction or discontinuation (24,25).

Several mechanisms have been proposed to participate in the characteristic intrarenal vasoconstriction, including excessive sympathetic nerve stimulation (26), altered eicosanoid metabolism (27–29), either decreased or unchanged activity of the renin-angiotensin system (30,31), alterations in calcium homeostasis by cyclosporine-enhanced transmembrane calcium influx and mobilization from intracellular stores (32,33). In addition, experimental studies suggest that cyclosporine causes exaggerated contractile responses in arteriolar smooth muscles and mesangial cells in the presence of vasoactive substances (34). In renal transplant recipients, calcium channel blockers have been reported to be beneficial in treating cyclosporine-induced impaired renal function and hypertension by counteracting renal vascular constriction or partially inhibiting cyclosporine-induced mesangium-cell contraction (35–37). The concomitant use of vasoactive drugs might therefore be an important determinant for the susceptibility to cyclosporine-induced renal dysfunction. In the investigation presented here, no relationship between cyclosporine-induced renal dysfunction and the use of calcium channel blockers, betablockers, angiotensin-converting enzyme inhibitors, and/or diuretics was detected.

The differences detected between responders and nonresponders could not be related to any of the studied recipient- or donor-specific covariates. However, the observed differences were rather discrete and several parameters known to be related with acute cyclosporine-induced renal dysfunction, such as serum potassium (38,39) and bilirubin levels (40), did not significantly increase in the responder group during the higher cyclosporine doses. More pronounced differences might have been observed with higher cyclosporine doses and/or a prolonged observation period. For ethical reasons, we did not take the risk to increase steady-state cyclosporine doses by more than 50% in these patients with stable renal function or to increase the number of subjects to be investigated. It should be noted that short-term cyclosporine administration often showed its nephrotoxic effects immediately after exposure (41–43).

In conclusion, this investigation demonstrates that the transplanted kidney itself and not the recipient determines the susceptibility to acute cyclosporine-induced toxicity.

## ACKNOWLEDGMENTS

We are grateful to all of our patients who participated in this study. It is anything but an obvious choice for a patient with a renal graft to accept the possibility of a transient decrease in renal function for the pure sake of science. This study was supported by Grant 32-3641.92 of The Swiss National Science Foundation.

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# Prevention of In Vitro Neutrophil–Endothelial Attachment through Shedding of L-Selectin by Nonsteroidal Antiinflammatory Drugs

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## Abstract

**The activation of the endothelial cells by extravascular stimuli is the key event in the extravasation of circulating leukocytes to target tissues. L-selectin, a member of the selectin family, is constitutively expressed by white cells, and is the molecule involved in the initial binding of leukocytes to activated endothelium. After activation, leukocytes rapidly release L-selectin from the cell surface, suggesting that the functional activity of this molecule is controlled in large part by its appearance and disappearance from cell surface. We have studied in a neutrophil-activated endothelial cell binding assay, the effect of different antiinflammatory drugs (steroidal and nonsteroidal) in the L-selectin–mediated interaction of neutrophils with activated endothelial cells. Some nonsteroidal antiinflammatory drugs (NSAIDs), such as indomethacin, diclofenac, ketoprofen, and aspirin, but not steroids, strongly inhibited the neutrophil–endothelial cell attachment. Furthermore, we also investigated the underlying mechanism of this functional effect. The expression of L-selectin on the neutrophil surface rapidly decreased in the presence of different NSAIDs, in a dose- and time-dependent manner, whereas no changes in the expression of other adhesion molecules such as CD11a, CD11b, CD31, or ICAM-3 (CD50) were observed. Interestingly, studies in vivo on healthy volunteers treated with physiological doses of indomethacin showed a significant decrease of L-selectin neutrophil expression. Only diclofenac induced an upregulation of CD11b expression, suggesting an activating effect on neutrophils. No enzyme release was observed upon treatment of neutrophils with different NSAIDs, indicating a lack of degranulatory activity of NSAIDs, with the exception of diclofenac. The downregulation of L-selectin expression was due to the rapid cleavage and shedding of the membrane L-selectin, as determined by both immunoprecipitation from $^{125}$I-labeled neutrophils, and quantitative estimation in cell-free supernatants. These results suggest that NSAIDs exert a specific action on adhesion receptor expression in neutrophils, which might account, at least in part, for the antiinflammatory activities of NSAIDs. (*J. Clin. Invest.* 1995. 95:1756–1765.)**

**Key words: adhesion molecules • L-selectin • neutrophils • nonsteroidal antiinflammatory drugs**

## Introduction

The aim of the inflammatory response is to eliminate deleterious agents and repair the damaged tissue. When the inflammation is persistent, it may be injurious to the host, causing severe tissue destruction and dysfunction as occurs in the joints of rheumatoid arthritis patients. The control of inflammation in human diseases characterized by an abnormal chronic inflammatory response is one of the challenges of the present medicine.

Leukocyte extravasation is essential in the inflammatory response. This process can be divided into three steps: initial interaction of leukocytes with activated endothelium (rolling), leukocyte activation with firm adhesion to endothelial cells and, finally, their extravasation into the surrounding tissues (1, 2). Several adhesion molecules are involved in the processes of adhesion and migration of leukocytes through vascular endothelium at sites of inflammation (1–3). Among them, L-selectin (Leu-8, LAM-1, MEL 14, or LECAM-1) has a key role in the initial attachment of circulating leukocytes to endothelium. This molecule is responsible for the rolling of leukocytes along the vascular walls, as a prerequisite to arrest movement and firm adhesion, thus allowing the interaction of other molecules (integrins) which account for *trans*-endothelial migration and cell extravasation to the target tissues (3–6).

L-selectin is a member of the selectin family of adhesion molecules that mediate adhesive interactions among endothelium, leukocytes, and platelets (7–9). Three members of the selectin family have been described which are differentially expressed (7, 8). P-selectin (CD62) is constitutively present in granules of platelets and endothelial cells and is rapidly translocated to the surface after specific stimuli (10–12). E-selectin (ELAM-1) is restricted to endothelial cells and is expressed after stimulation with certain cytokines (13). E-selectin and P-selectin mediate endothelial interactions with neutrophils, monocytes, and some lymphocytes (7, 8). L-selectin is a highly glycosylated protein of 95–105 kD on neutrophils and 74 kD on lymphocytes that is constitutively expressed by most of leukocytes (14, 15). This adhesion molecule is involved in leukocyte–endothelial cell interactions by recognition of carbohydrate determinants found in several endothelial cell ligands, including GlyCAM-1 (16), CD34 (17), MAdCAM-1 (18) and P- and E-selectins (19). Interestingly, L-selectin is rapidly shed after neutrophil activation both in vitro (20–22) and in vivo (23, 24). A soluble form of L-selectin that retains functional activity has been detected in sera from normal human donors (25). In this regard, we have also described a soluble form of L-selectin in synovial fluid from different inflammatory joint diseases (24).

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Nonsteroidal antiinflammatory drugs (NSAIDs)[1] are a heterogeneous group of chemical compounds very commonly prescribed for the control of inflammatory human diseases (26). The precise mechanism of action of NSAIDs is not completely understood. It has been proposed that the major mechanism of action of these drugs is the inhibition of prostaglandin synthesis (27). However, this inhibitory activity does not account for all antiinflammatory effects of NSAIDs. Additional effects of NSAIDs have been described (28, 29), but none of them explain the entire spectrum of their antiinflammatory activities.

To search for functional and molecular targets of NSAIDs, we examined the possible effect of different antiinflammatory drugs (steroidal and nonsteroidal) at the first step of neutrophil extravasation, the L-selectin–mediated interaction of neutrophils with activated endothelial cells. We found that most NSAIDs, but not steroids, strongly inhibited this event. Furthermore, we also investigated the mechanism underlying this functional effect, and we found that it is due to the loss of L-selectin expression in neutrophils through a shedding mechanism.

## Methods

*Antibodies and reagents.* The following mAbs were used: Bear-1 anti-CD11b, D3/9 anti-CD45, TP1/40 anti-CD11a, HP2/19 anti-ICAM-3, TP1/15 anti-CD31, and P3X63 myeloma culture supernatant as a negative control (30, 31). The Leu-8 anti–L-selectin mAb was purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). LAM1-3 and LAM1-12 anti—L-selectin mAbs (32) were kindly provided by Dr. T. Tedder (Duke University, Durham, NC).

TNF-$\alpha$ (sp act $3.2 \times 10^7$ U/mg) was purchased from Wichem International (Vienna, Austria). Aspirin, indomethacin, diclofenac, ketoprofen, piroxicam, dexamethasone, and PMA, were purchased from Sigma Chemical Co. (St. Louis, MO).

*Isolation and treatment of neutrophils.* Neutrophils were isolated from peripheral blood by Ficoll–Hypaque density gradient centrifugation (Pharmacia Diagnostics AB, Uppsala, Sweden), followed by sedimentation at 1 *g* in 1.3% dextran (Sigma Chemical Co.) at room temperature. The neutrophil-enriched fraction was further purified by hypotonic lysis of erythrocytes, giving a purity > 98%. The cells were resuspended on Hepes-glucose buffer (glucose 150 mM NaCl, 5 mM KOH, 10 mM Hepes, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.5 mM, pH: 7.5). The experiments were carried out in 15 ml disposable polypropylene tubes (Falcon Labware, Oxnard, CA).

Neutrophils were incubated in Hepes-glucose buffer with the different drugs, TNF-$\alpha$ or PMA at times and temperatures indicated. In some assays, 4 g/liter of HSA was included in the buffer. The dose of each drug was chosen according to their potency of antiinflammatory activity. The pH of the aspirin solution ranged between 6.3 and 7.2, depending on drug concentrations (1–0.125 mg/ml). These pH values did not alter the L-selectin neutrophil surface expression. Cell viability, estimated after each treatment by trypan blue exclusion, was > 95%.

*Flow cytometry analysis.* Flow cytometry analysis was performed in a FACScan® cytofluorometer (Becton Dickinson Immunocytometry Systems). Resting and stimulated neutrophils were incubated with hybridoma culture supernatants followed by washing and labeling with an FITC-labeled goat anti–mouse Ig (Dakopatts, Slastrup, Denmark). Linear and logarithmic immunofluorescence values were obtained in each experiment and the fluorescence produced by the myeloma P3X63 supernatant was considered as background. A total of 5,000 cells was analyzed from each sample adjusting the fluorescence gain so that ~ 5% of the cells of the sample with greatest fluorescence were positive in the highest fluorescence channel. The results are displayed either in a linear or logarithmic scale of fluorescence intensity and presented in some figures as the percentage of variation of baseline mean fluorescence intensity (MFI) (%ΔMFI) of total cells.

$$\%\Delta MFI = [(\text{absolute } MFI_{NSAIDs}/\text{absolute } MFI_{medium}) - 1] \times 100. \quad (1)$$

*Radiolabeling, immunoprecipitation, and electrophoresis.* Cell surface proteins of $6 \times 10^7$ neutrophils from a healthy donor were radiolabeled with $Na^{125}I$ in a solution with chloroglycoluril (Iodo-Gen; Pierce Chemical Co., Rockford, IL), washed twice with PBS and, finally, resuspended on Hepes-glucose buffer with 1% BSA. Radioiodinated cells were either incubated with NSAIDs or maintained with buffer during 15 min at 37°C. The cells and the cell-free supernatants were separated by centrifugation. The cell-free supernatants were supplemented with 1 mM PMSF and 1% Triton X-100. Cell pellets were lysed with PBS containing 1% Triton X-100, 1% hemoglobin, and 1 mM PMSF. After overnight dialysis against PBS, samples were precleared with protein A from *Staphylococcus aureus* coupled to Sepharose. For immunoprecipitation, an equal amount of radioactivity of each sample was mixed with 100 $\mu$l of mAb containing hybridoma culture supernatant. Immune complexes were isolated by the addition of 100 $\mu$l of the 187.1 rat anti–mouse $\kappa$-chain monoclonal antibody, followed by 30 $\mu$l of protein A coupled to Sepharose. Immunoprecipitates were processed as previously described (33), and samples were subjected to 10% SDS-PAGE and autoradiography with enhancing screens. A prestained molecular weight standard mixture (Sigma Chemical Co.) was used as molecular weight markers.

*Neutrophil–endothelial cell attachment assay.* Human umbilical vein endothelial cells (HUVEC) were isolated and grown in M199 culture medium supplemented with 10% FCS, endothelial cell growth factor (50 $\mu$g/ml, Biochemical Technologies Inc., Stoughton, MA) and porcine intestinal heparin (50 $\mu$g/ml, Sigma Chemical Co.) as described (34). Endothelial cells (passage 2–3) were grown to confluence on gelatin (0.1%)-precoated 24-well plates and stimulated with either TNF-$\alpha$ (20 ng/ml) or medium alone at 37°C. Cell monolayers were carefully washed and incubated at 4°C for 15 min with 200 $\mu$l of RPMI 1640 medium containing 10% FCS. Then, $1 \times 10^6$ neutrophils in 100 $\mu$l were added. Previously, neutrophils were incubated with different NSAIDs for the time and dose indicated. Samples of untreated neutrophils were additionally incubated with the anti–L-selectin LAM-1-3 and LAM-1-12 mAbs for 15 min at 4°C. After 30 min of incubation at 4°C with rotation at 64 rpm, wells were washed (5×) with cold PBS and fixed in 1% of glutaraldehyde in PBS.

The number of bound neutrophils was determined by direct counting on an inverted microscope. Inhibition of neutrophil adhesion was calculated using the level of attachment to unstimulated HUVEC as the baseline, and the level of neutrophils attachment to TNF-$\alpha$–stimulated HUVEC as the maximal value.

*Enzymatic activity in supernatants from NSAIDs-treated neutrophils.* Resting neutrophils were resuspended at $5 \times 10^6$ cells/ml in 1 ml of Hepes-glucose buffer and incubated in the presence of distinct agents for 15 min at 37°C. Then, cells were pelleted by centrifugation and supernatants were assayed for marker activities. Enzyme release was determined as the percentage of total enzyme units in the supernatant. Gelatinase, lysozyme, and *N*-Acetyl-$\beta$-glucosaminidase were assayed as described (30, 35).

*sL-selectin ELISA.* The ELISA used to quantitate sL-selectin in the supernatant of NSAIDs-treated neutrophils was generously provided by Bender MedSystem (Vienna, Austria).

*Studies in vivo of L-selectin neutrophil expression.* Neutrophils from seven healthy volunteers taking per os indomethacin at 25 mg, three times a day, were analyzed for cell surface expression of different adhesion molecules by flow cytometry. Peripheral blood samples were obtained just before starting indomethacin ($t = 0$), and 60–90 min after the ingestion of the third ($t = 24$ h) and sixth doses ($t = 48$ h). To minimize the daily variations of fluorescence staining and FACScan sensitivity, the fluorescence intensity (FI) corresponding to CD11b and

1. *Abbreviations used in this paper:* FI, fluorescence intensity; HUVEC, human umbilical vein endothelial cells; MFI, mean FI; NSAIDs, nonsteroidal antiinflammatory drugs.

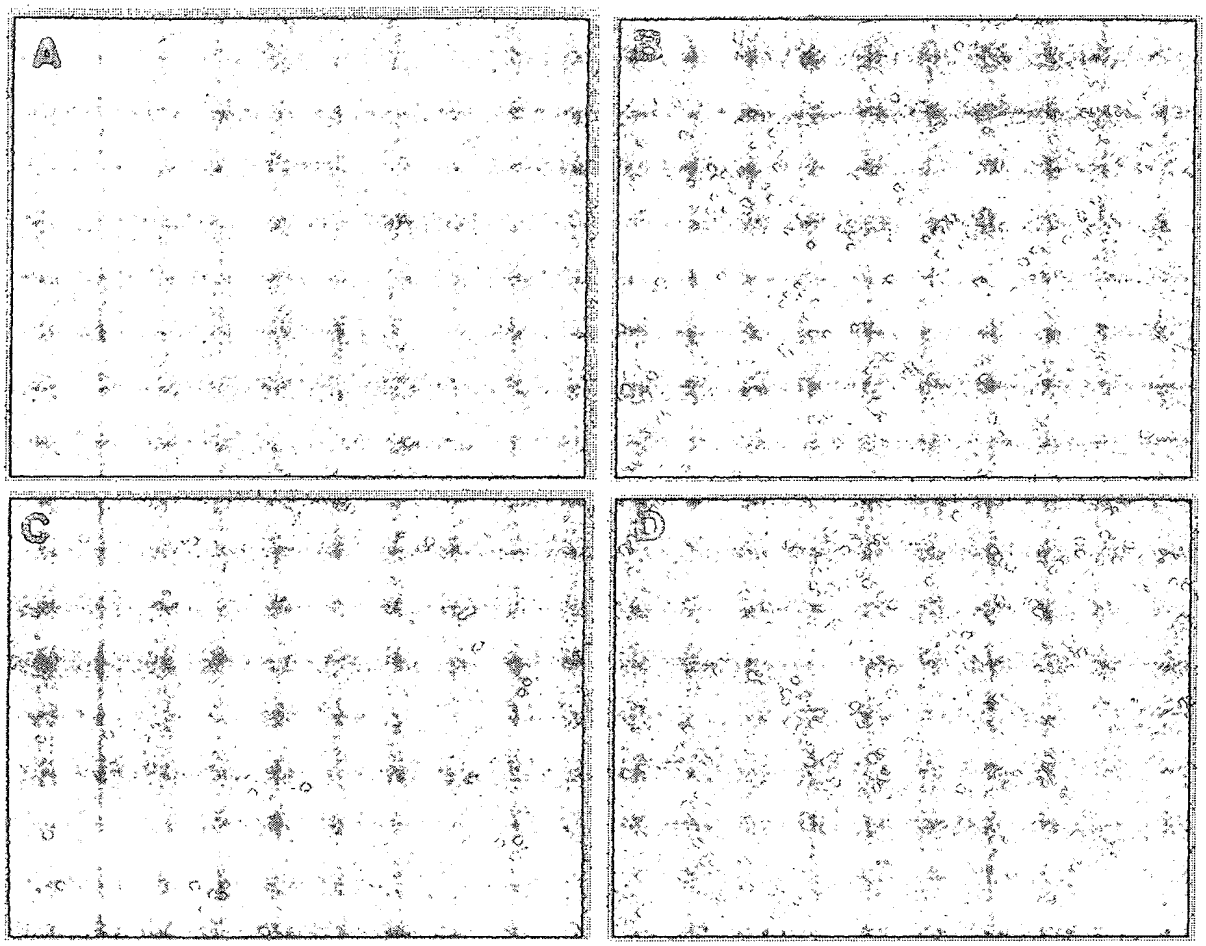

*Figure 1.* Effect of various antiinflammatory drugs on neutrophil attachment to human endothelial cell monolayers. Confluent endothelial cell monolayers cultured for 6 h in the presence of 20 ng/ml TNF-$\alpha$ were used for neutrophil adhesion assays. A significant adhesion of neutrophils was evident to the activated endothelium (*B*) (baseline adhesion), but not to unstimulated cell monolayers (*A*). Preincubation of neutrophils with anti-LAM1-3 significantly decreased the basal adhesion (*C*). In contrast, the anti-LAM1-12 did not show a significant effect in the neutrophil–endothelial attachment assay (*D*). The adhesion to activated endotelium was nearly completely abrogated by preincubating the neutrophils with indomethacin (0.2 mg/ml) (*E*) and diclofenac (0.2 mg/ml) (*F*), whereas the adhesion of piroxicam (0.2 mg/ml) pretreated neutrophils (*G*) was found similar to control (*B*).

L-selectin expression in each sample was related to that of CD11a, whose expression remains stable during neutrophil activation and NSAIDs treatment. The results are presented as relative fluorescence intensity (RFI).

$$\text{RFI} = (\text{CD11b or L-selectin FI} - \text{P3X63 FI}) \times 100/(\text{CD11a FI} - \text{P3X63 FI}). \quad (2)$$

Student's *t* test for paired samples was used to compare the results.

## Results

*Inhibition of L-selectin–mediated neutrophil adhesion to endothelial cells by NSAIDs.* To study the initial event of inflammation, we analyzed in vitro the binding of neutrophils to HUVEC under nonstatic conditions. In a first step, we studied the effect of the endothelial activation state on the neutrophil attachment. Only few neutrophils were able to bind to unstimulated endothelium (Fig. 1 *A*). However, when the endothelium was activated with TNF-$\alpha$ to induce the expression of the L-selectin ligand(s), a significant number of neutrophils attached to endothelial cells (Fig. 1 *B*). In agreement with results previously reported by other authors (36), this binding was preferentially mediated by the L-selectin, since anti–L-selectin LAM1-3 mAb reduced 70% in this interaction (Fig. 1 *C*). On the other hand, the anti–L-selectin LAM 1–12 mAb did not inhibit the neutrophil–endothelial cell interaction (Fig. 1 *D*).

Next, we assayed the effect of different antiinflammatory drugs (nonsteroidal and steroidal) on the L-selectin–mediated neutrophil–endothelial interaction. The concentration of each drug tested was proportional to their antiinflammatory potency. When neutrophils were preincubated for 15 min at 37°C either

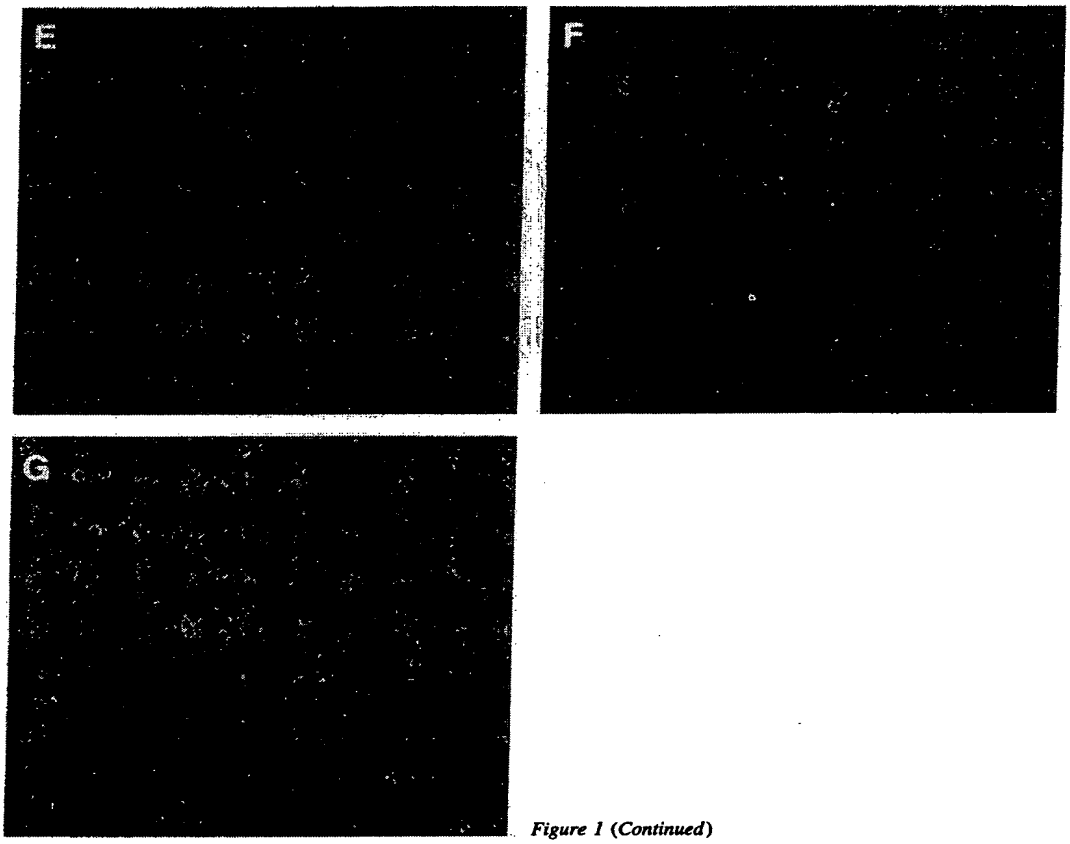


*Figure 1 (Continued)*

with indomethacin (0.2 mg/ml), diclofenac (0.2 mg/ml), or aspirin (1 mg/ml), the adhesion of neutrophils to endothelium was almost completely inhibited (Fig. 1, *E* and *F*, data not shown). Inhibitory effects were also observed at lower doses, in the range of 10–50 μg/ml for diclofenac or indomethacin, and 0.3 mg/ml for aspirin. Similar results were obtained when neutrophils were treated with drugs in the presence of physiologic concentrations (4 g/liter) of human serum albumin. Under these conditions, slightly higher drug concentrations were required (not shown). In contrast, piroxicam (0.2 mg/ml) and dexamethasone (0.2 mg/ml) did not inhibit the neutrophil adhesion to endothelial cells (Fig. 1 *G*, data not shown).

*L-Selectin neutrophil expression is downregulated by NSAIDs.* To determine the mechanism of inhibition of neutrophil-endothelial cell interaction by NSAIDs, we have studied the possible effect of these drugs on the cell surface expression of L-selectin. Flow cytometry analysis of neutrophils treated with indomethacin, diclofenac, piroxicam, ketoprofen, and aspirin in the same conditions than the adhesion experiments, showed that the cell surface expression of L-selectin was downregulated by all NSAIDs except piroxicam (Fig. 2). Diclofenac and indomethacin showed a stronger effect than ketoprofen and aspirin on L-selectin downregulation (Fig. 2). In contrast, dexamethasone, a potent synthetic steroid, and hydrocortisone, the main physiologic glucocorticoid, did not modify L-selectin expression at concentrations as high as 0.2 mg/ml (Fig. 2, and data not shown). When these experiments were performed at 4°C, no modification of the basal expression of L-selectin was observed (data not shown).

The effect of NSAIDs on L-selectin expression on neutrophils was both dose- and time-dependent. Fig. 3 *A* shows a dose-response experiment in which in a decreasing order of potency, diclofenac, indomethacin, and aspirin were effective on the downregulation of L-selectin expression, while piroxicam caused no effect. L-selectin downregulation required slightly higher drug concentrations when assays were performed in the presence of human serum albumin. Under these conditions, doses > 50 μg/ml of diclofenac or indomethacin, and 0.3 mg/ml of aspirin were required to induce the effect. Kinetics studies using 40 μg/ml of diclofenac, indomethacin, and piroxicam and 0.5 mg/ml of aspirin, indicated that diclofenac has the fastest effect by decreasing the L-selectin expression after 5 min of treatment (Fig. 3 *B*). Indomethacin was also active at this concentration, but 60 min were required for displaying a significant effect. Aspirin only had a mild action and piroxicam did not modify the basal expression of L-selectin by periods of time as long as 120 min (Fig. 3 *B*).

The expression of other cell adhesion molecules, constitutively expressed by neutrophils, such as CD31 (PECAM-1), CD50 (ICAM-3), and CD45, was not altered upon exposure to the different NSAIDs studied. A representative experiment of the effect of indomethacin (0.2 mg/ml at 37°C during 15

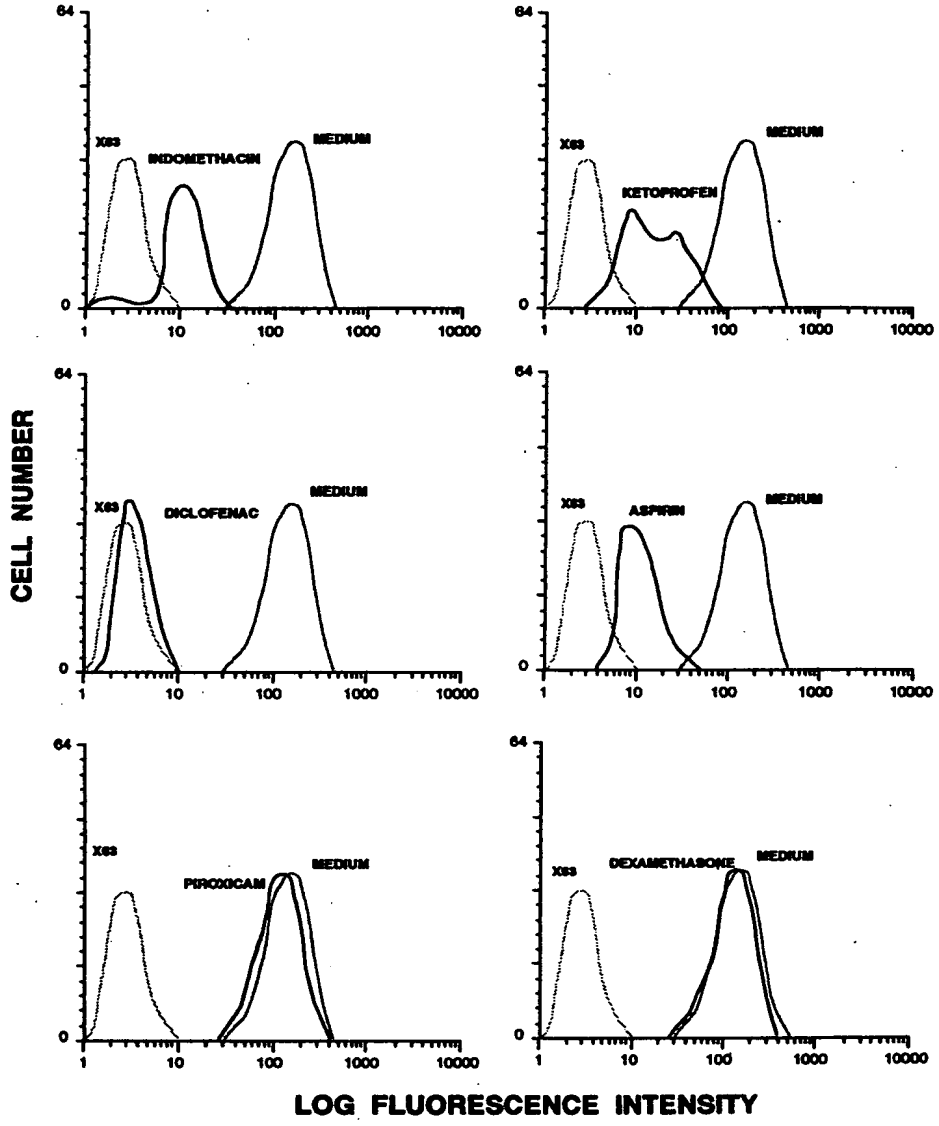


*Figure 2.* Effect of antiinflammatory drugs on cell surface expression of L-selectin by human neutrophils. Neutrophils were incubated for 15 min at 37°C in medium alone or in the presence of 0.2 mg/ml indomethacin, diclofenac, and piroxicam, 1 mg/ml aspirin, 2 mg/ml ketoprofen, and 0.2 mg/ml dexamethasone (*solid line*). In each histogram is also displayed the basal expression of the L-selectin (*medium*) and the negative control of immunostaining (P3 X63). One representative experiment ($n = 5$) is shown.

min) on the expression of L-selectin, ICAM-3, CD31, and CD45 on neutrophils is shown in Fig. 4.

These data indicate that some NSAIDs induce a downregulation of L-selectin expression on neutrophils, that occurs in a time- and dose-dependent manner.

*Effect of NSAIDs on neutrophil activation.* L-selectin is rapidly downregulated from the neutrophil cell surface upon cell activation (20–22). Therefore, we analyzed the possibility that NSAIDs could induce neutrophil activation. It has previously been shown that the expression of CD11b and CD45 is upregulated upon neutrophil activation (30, 37). The results shown in Fig. 5 *A* clearly indicate that most of NSAIDs tested did not modify CD11b baseline expression upon 15 min of cell treatment. However, diclofenac increased CD11b cell surface expression. The expression of CD11a, that is not affected by cell activation, was used as control (Fig. 5 *A*). Kinetics experiments clearly showed that diclofenac, but not indomethacin, piroxicam, or aspirin, clearly enhanced CD11b cell surface expression in a time- and dose-dependent manner (Fig. 5, *B* and *C*, respectively).

Parallel experiments were conducted to examine the degree of degranulation of the different types of intracellular granules described in human neutrophils (30, 35). As shown in Table I, neither gelatinase (a marker for tertiary granules) nor lysozyme (a marker for specific granules) were secreted upon cell preincubation with different NSAIDs, with the exception of diclofenac. TNF-$\alpha$ and diclofenac induced a significant release of gelatinase and a slight release of lysozyme. As these agents augmented the cell surface expression of CD11b (Fig. 5), these data further support the location of this antigen in gelatinase-

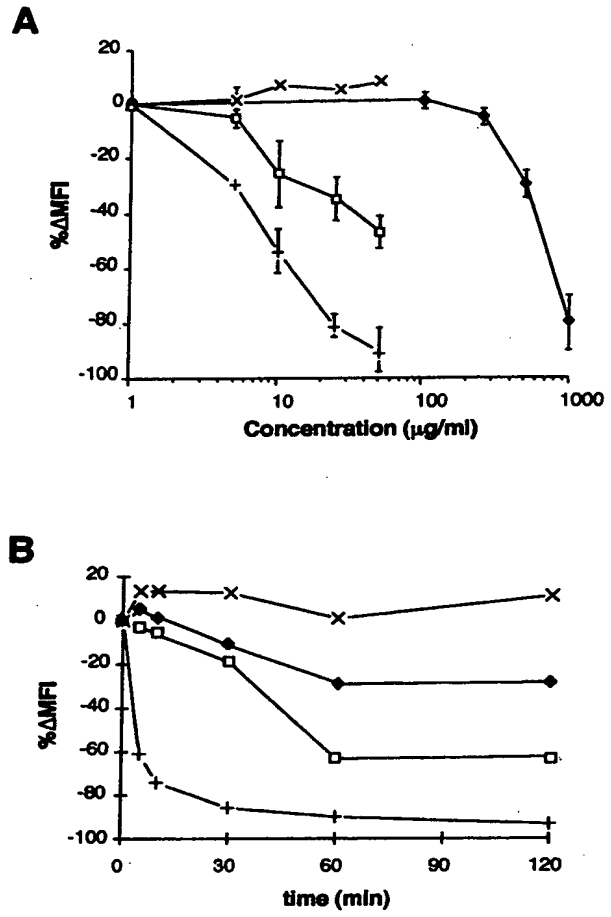


*Figure 3.* Dose response and time course of the effect of NSAIDs on L-selectin neutrophil expression. (*A*) Dose response of the L-selectin downregulation induced by NSAIDs on normal neutrophils. Cells were cultured for 1 h in the presence of different concentrations of various NSAIDs. The dots indicate the average of three different experiments and the error bars indicate the ranges —□—, indomethacin; —+—, diclofenac; —×—, piroxicam; and —◇—, aspirin. (*B*) Kinetics of the effect NSAIDs on normal neutrophils. Cells were cultured in the presence of 40 μg/ml of indomethacin, diclofenac, and piroxicam and 0.5 mg/ml aspirin until 120 min. The percentage of variation of the L-selectin expression (%ΔMFI) was related to the expression by cultured cells in medium in each time.

containing granules (30). As control, cell stimulation with the phorbol ester PMA induced a high secretion of gelatinase as well as the partial release of lysozyme. No secretion of *N*-acetyl-β-glucosaminidase (a marker for azurophilic granules), even under the highest stimulatory condition, was observed (Table I).

These results suggest that neutrophil activation is not involved in the mechanism of regulation of L-selectin expression by indomethacin, ketoprofen, and aspirin. However, it is possible that the downregulation of L-selectin induced by diclofenac is, at least in part, mediated by a cell activation–dependent mechanism.

*In vivo effect of indomethacin on neutrophil L-selectin expression.* To ascertain the relevance of the above described observations, in vivo experimentation with human healthy volunteers was carried out. A preliminary kinetics study with two individuals taking physiological amounts of indomethacin (25 mg, three times a day, during 4 d), showed a significant neutrophil L-selectin downregulation beginning at 24 h, that was maintained during treatment (data not shown). Therefore, we decided to undertake the study of the neutrophil expression of L-selectin, CD11b, and CD11a in seven different volunteers 24, and 48 h after starting the administration protocol of indomethacin. The results obtained are shown in Fig. 6. A mean decrease of 38% (95% CI 21%, 55%) of L-selectin expression level was observed after 24 h, that was slightly lower by 48 h. In contrast, CD11b and CD11a neutrophil expression did not significantly change.

These results confirm in vivo, the in vitro observations of L-selectin downregulation with no significant change of CD11b after neutrophil exposure to indomethacin. Furthermore, they also indicate that physiological amounts of this NSAID caused a noticeable loss of neutrophil L-selectin.

*Treatment of human neutrophils with NSAIDs causes shedding of L-selectin.* L-selectin is constitutively expressed at the cell surface of neutrophils and it is rapidly shed after either cell activation (20–22) or activation-independent chemical cross-linking (38). Therefore, we investigated whether the NSAIDs exert their blocking effects on neutrophil–endothelial cell adhesion by the removal and shedding of L-selectin.

To this end, neutrophils were treated with different NSAIDs and then immunoprecipitation of L-selectin from both cell lysates and cell-free supernatants was performed. L-selectin was immunoprecipitated from the cell-free supernatant from neutrophils that have been treated with either indomethacin, diclofenac, or aspirin, but not from their corresponding cell lysates (Fig. 7 *A*). In contrast, no CD11b or CD31 antigens were immunoprecipitated from cell-free supernatants of NSAIDs-treated neutrophils (Fig. 7, *B* and *C*). Furthermore, the amounts of sL-selectin detected with an ELISA in the cell-free supernatants of neutrophils treated with indomethacin and diclofenac were similar to that observed in TNF-α–activated cells, and they varied between 1.6 to 2.2 ng/ml per $10^6$ cells (Fig. 8). In contrast, lower amounts of sL-selectin (0.5 ng/ml per $10^6$ cells) were found in the supernatant fluid of neutrophils incubated with piroxicam or medium (Fig. 8). These data demonstrate that some NSAIDs induce a selective and activation-independent shedding of L-selectin in neutrophils.

## Discussion

The endothelium plays an essential role in inflammation, providing the key signals for leukocyte migration to extravascular tissues. In normal conditions, the circulating leukocytes have continuous random contacts with endothelial cells, but no firm interactions occur. When a tissue is injured, the endothelial cells are activated by extravascular stimuli inducing the expression of a wide array of cell surface adhesion molecules. These molecules mediate a close receptor–counterreceptor interaction with circulating leukocytes, allowing the adhesion and the extravasation of leukocytes through vascular endothelium at sites of inflammation. The initial interaction with endothelium facilitates the rolling and arrest of circulating leukocytes on the endothelium surface. One of the leukocyte molecules involved in this

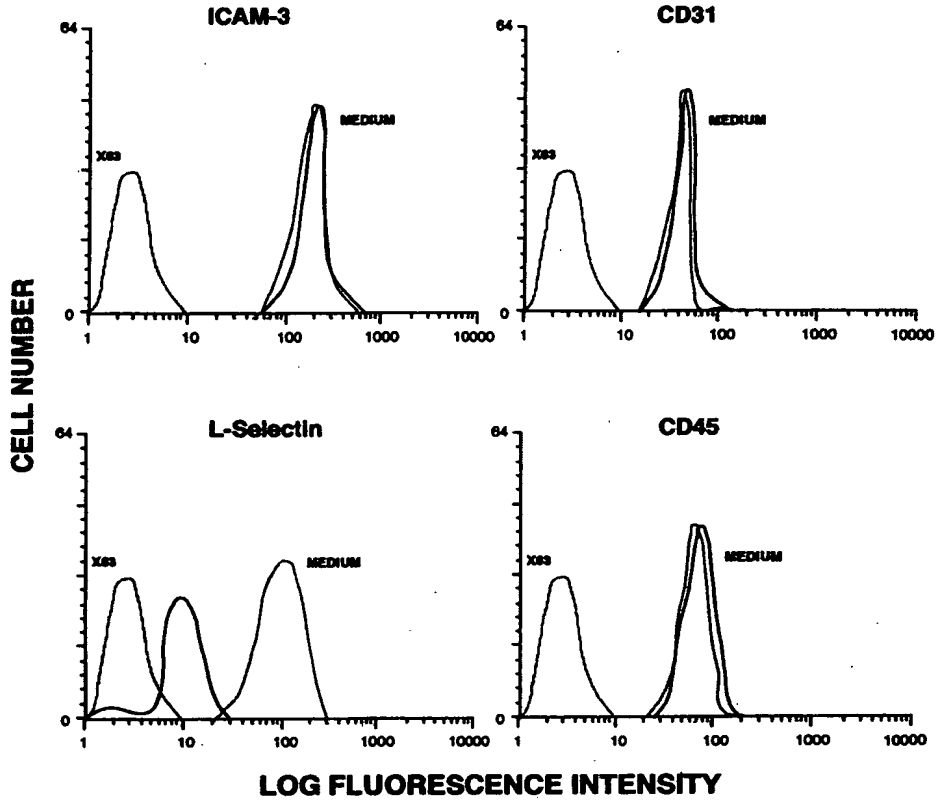


*Figure 4.* Effect of indomethacin on neutrophil surface expression of ICAM-3, CD31, and CD45. Neutrophils were incubated for 15 min at 37°C in the presence of 0.2 mg/ml indomethacin (*solid line*). In each histogram is displayed the basal expression of the cell surface protein (*medium*) and the negative control of immunostaining (P3 X63).

initial interaction is the L-selectin, a member of the selectin family which recognizes carbohydrates expressed by the activated endothelium (34, 39–42). Nowadays, research teams have undertaken strategies towards treating inflammation by blocking the selectin-mediated functions (43–45). In this therapeutic approach, it is assumed that if the initial L-selectin–mediated leukocyte rolling could be blocked, the following events involved in the leukocyte migration to the tissue would not occur and the inflammatory response would be alleviated.

The aim of this study was to search for drugs with the capability of blocking the binding of neutrophils to endothelial cells and to determine their mechanisms of action. To this end, a neutrophil-HUVEC attachment assay was used to study the first step of neutrophil migration. To simulate the blood flow, the experiments were performed under nonstatic conditions (36). In these assays, the binding of neutrophils to activated HUVEC was mediated by the L-selectin as it occurs during both in vitro and in vivo rolling at physiologic cell flow rates (4–6). Initially, we studied the effect on neutrophils of drugs with antiinflammatory properties such as glucocorticoids and NSAIDs. The preincubation of neutrophils with different NSAIDs produced a dramatic abrogation of the neutrophil attachment to activated HUVEC. In contrast, dexamethasone showed no effect pointing out that nonsteroidal drugs had a different antiinflammatory mechanism of action. This inhibitory effect on neutrophil adhesiveness is not common to all NSAIDs; piroxicam, an enolic acid–derived NSAID, did not show any inhibitory effect on the L-selectin–mediated neutrophil-HUVEC attachment assay. Preliminary evidence indicates that piroxicam interferes with the process of neutrophil activation and degranulation, induced by stimuli like TNF-$\alpha$ or the chemoattractant peptide FMLP (Díaz-González, unpublished observations). Although the major mechanism of action of NSAIDs is well established to be ciclooxigenase inhibition and interference with synthesis of prostaglandins (27), our results point out differences in the effects and mode of action among various NSAIDs, in accordance with previous reported work (46). In this regard, it has recently been described that certain NSAIDs, as sodium salicylate and aspirin, inhibit NF-KB-dependent transcription activity (47). A chemical structure common to aspirin, indomethacin, diclofenac, and ketoprofen, but absent in piroxicam, might be responsible for the L-selectin shedding in neutrophils; this datum might be important for the future development of new antiinflammatory drugs that interfere with L-selectin–mediated function.

The relevant role of L-selectin in the nonstatic binding assay used by us, suggested that NSAIDs could affect the neutrophil L-selectin expression. In this regard, we have shown herein that those drugs which inhibit the neutrophil-HUVEC attachment, also downregulate the L-selectin cell surface expression in a dose- and time-dependent manner. However, neither piroxicam nor dexamethasone modified the basal expression of this selectin in neutrophils. Activation of neutrophils uniformly results in downregulation of L-selectin expression (20–22) as well as in upregulation of both CD11b/CD18 and CD45 expression (22, 30, 37). Nevertheless, the basal expression of CD11b and

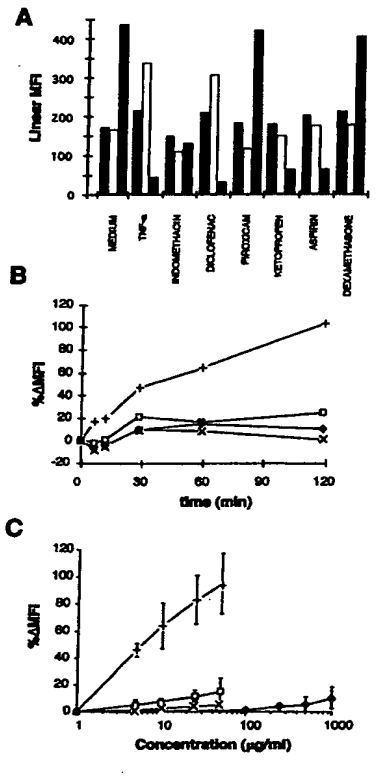


*Figure 5.* Effect of NSAIDs on neutrophil CD11b cell surface expression. (*A*) Linear mean fluorescence intensity of CD11a (■), CD11b (□), and L-selectin (■) after incubation with 0.2 mg/ml indomethacin, diclofenac, and piroxicam, 2 mg/ml ketoprofen, 1 mg/ml aspirin and 0.2 mg/ml dexamethasone for 15 min at 37°C. A downregulatory effect of indomethacin, diclofenac, ketoprofen, and aspirin on L-selectin expression is shown. Only diclofenac induced a simultaneous upregulation of CD11b. (*B*) Kinetics of the effect of NSAIDs on the surface expression of CD11b. The drugs were used at the same doses as indicated in (*A*). —□—, indomethacin, —+—, diclofenac; —×—, piroxicam; and —◇—, aspirin. (*C*) Dose response of CD11b expression under same experimental conditions as in Fig. 3 *A*. The dots indicate the mean and the error bars indicate the ranges.

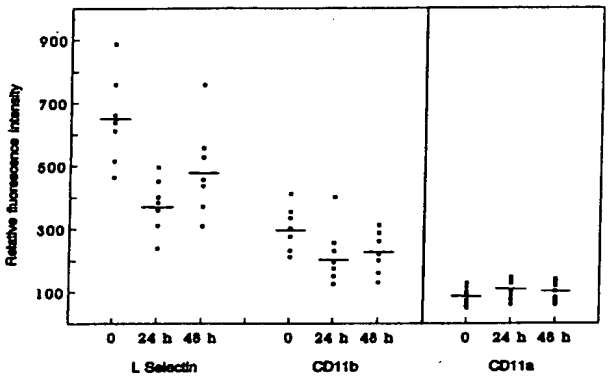


*Figure 6.* Expression of L-selectin and CD11b adhesion molecules on neutrophils from healthy volunteers during administration of indomethacin. L-selectin neutrophil levels decreased significantly at 24 h ($P < 0.01$), and 48 h ($P < 0.001$) after indomethacin treatment; CD11b expression had no significant variation. Levels of CD11a expression are presented as absolute immunofluorescence intensity, showing the absence of changes during treatment.

CD45 was unaffected by most of the drugs tested, suggesting that neutrophils are not activated by NSAIDs, and that the downregulation of L-selectin is mediated by an activation-independent mechanism. This is further supported by the lack of neutrophil degranulatory activity of NSAIDs as determined by granule enzyme release. Only diclofenac induced a significant increase of the expression of both CD11b and CD45 glycoproteins, as well as a partial neutrophil degranulation suggesting that this drug is capable, at least in part, to downregulate L-selectin expression through a mechanism that involves neutrophil activation. Accordingly, it has been reported that cross-linking of the L-selectin is also an activation-independent mechanism of downregulation of this molecule (38). In addition, high levels of soluble L-selectin have been found in plasma from normal subjects (25), suggesting that this soluble L-selectin is released from leukocytes through a mechanism in which cell activation is not involved.

*Table I. Granule Marker Release in Human Neutrophils by Different NSAIDs*

| | Enzyme release | | |
|---|---|---|---|
| | Gelatinase | Lysozyme | NAβGase |
| Control | 21.3±2.1 | 5.9±1.7 | 2.5±0.5 |
| PMA | 63.7±10.7 | 33.6±2.9 | 3.1±0.6 |
| TNF-α | 53.8±8.8 | 8.9±1.6 | 3.0±0.2 |
| Diclofenac | 38.9±8.6 | 8.1±2.8 | 2.5±0.3 |
| Piroxicam | 16.0±5.4 | 1.8±0.9 | 2.9±0.6 |
| Ketoprofen | 21.6±1.3 | 4.1±1.9 | 2.2±0.2 |
| Aspirin | 16.1±2.2 | 5.0±2.1 | 2.4±0.4 |
| Indometacin | 23.8±3.3 | 6.1±2.0 | 3.3±0.8 |

Data are shown as percentage of total cellular enzyme activity released to the extracellular medium. Total cell enzyme was measured in resting cells disrupted by treatment with 0.1% Triton X-100 and/or by thawing/freezing. Values are shown as mean±SE of three independent determinations. NAβGase, *N*-acetyl-β-glucosaminidase.

It has been reported that the loss of L-selectin in activated neutrophils takes place through a proteolytic shedding of the molecule and that none of the known protease inhibitors inhibit the shedding of L-selectin (14, 20, 22, 25). We have demonstrated that the loss of L-selectin induced by NSAIDs is also due to the shedding of the molecule from the cell surface, as confirmed by both immunoprecipitation and quantitative estimation of a soluble form of this molecule in cell-free supernatants of neutrophils treated with those drugs. It is tempting to speculate that a selective activation of a specific protease might account for this phenomenon. However, the mechanism of this NSAIDs-mediated L-selectin activation–independent shedding remains to be clarified. Although an inherent proteolytic activity of NSAIDs themselves on L-selectin cannot be completely ruled out, the observation that this phenomenon does not occur at 4°C supports the presence of an inducible cellular protease.

The concentrations of different NSAIDs required in our in vitro studies for the L-selectin shedding from neutrophil surface appear to be higher than those described to exert its antiinflammatory effects in vivo (48). Interestingly enough, when healthy donors were treated with physiological doses of indomethacin, a significant loss of L-selectin neutrophil expression was detected, with no change in CD11a or CD11b expression. This decrement of neutrophil L-selectin in vivo is remarkable taking into account that it is observed in the cell population of blood neutrophils, that is subjected to a rapid and continuous renewal. On

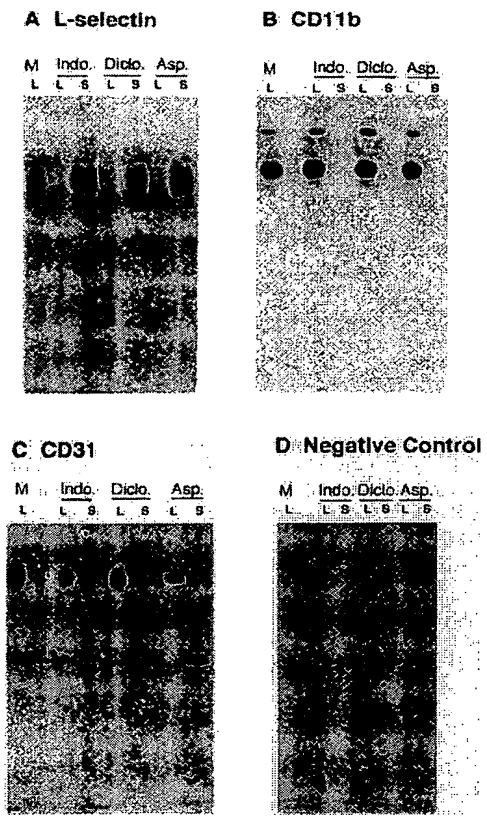


*Figure 7.* Immunoprecipitation of L-selectin, CD11b, and CD31 from cell lysates (*L*) and cell-free supernatants (*S*). Untreated (*Medium*) and NSAIDs-treated neutrophils, with the doses and time used in the neutrophil-endothelial attachment assay (see Methods) were immunoprecipitated with the following mAb: anti-LAM-1 Leu-8, anti-CD11b Bear-1, anti-CD31 TP1/15, and P3 X63 as negative control. Indo, indomethacin; Diclo, diclofenac; Asp, aspirin.

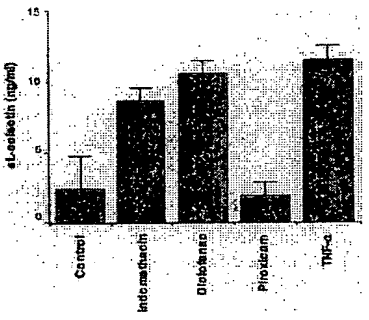


*Figure 8.* Quantification of neutrophil-shed L-selectin induced by NSAIDs. Neutrophils ($5 \times 10^6$) were incubated in medium alone (*control*) or in the presence of indomethacin (0.2 mg/ml), diclofenac (0.2 mg/ml), piroxicam (0.2 mg/ml), and TNF-$\alpha$ (20 ng/ml) for 15 min at 37°C. Culture medium samples were centrifuged, and supernatant fluids were tested for sL-selectin by a specific ELISA. The concentration of sL-selectin was calculated by comparing the OD with those obtained with recombinant sL-selectin standards, using a linear regression analysis. Values represent the mean(ng/ml)±SE obtained in duplicate determinations for each sample. The results are representative of two experiments.

the other hand, indomethacin does not appear to activate neutrophils in vivo as determined by its failure to upregulate CD11b expression. However, it is difficult to establish a strict correlation between our in vitro observations and in vivo behavior of these types of drugs which is depending not only on plasma levels, but also on different factors as preferential tissue accumulation, plasma half-lives, hepatic biotransformation, and enterohepatic recycling of NSAIDs. On the other hand, all these pharmacologic parameters are different for the various NSAIDs groups (46). Nevertheless, it is conceivable that the action of these drugs on L-selectin neutrophil surface expression might account, at least in part, for antiinflammatory activity of NSAIDs.

In summary, we have shown that some NSAIDs inhibit neutrophil attachment to HUVEC in vitro through a rapid L-selectin cell shedding. These results suggest a specific mechanism of regulation of neutrophil adhesion functions by NSAIDs, which open a door for the development of new antiinflammatory drugs based on a selective L-selectin shedding activity. Therefore, the measurement of neutrophil L-selectin cell surface expression, could be a rapid and reproducible primary assay in the search of drugs with antiinflammatory activity.

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# Nonsteroidal Anti-Inflammatory Drugs: Effects on Kidney Function

*Andrew Whelton, MD, FACP, FCP, and Cindy W. Hamilton, PharmD*

*Nonsteroidal anti-inflammatory drugs (NSAIDs) are capable of inducing a variety of renal function abnormalities, particularly in high-risk patients with decreased renal blood perfusion who depend on prostaglandin synthesis to maintain normal renal function. Fluid retention is the most common NSAID-related renal complication, occurring to some degree in virtually all exposed individuals; however, clinically detectable edema occurs in less than 5% of patients and is readily reversible on discontinuation of the NSAID. Other electrolyte complications, notably hyperkalemia, are seen infrequently and occur in specific at-risk patients. The next most worrisome complication is acute deterioration of renal function, which occurs in high-risk patients and is also reversible. Nephrotic syndrome with interstitial nephritis is a rare problem of NSAID use and is reversible. Papillary necrosis is the only permanent complication of NSAIDs and is very rare. Altogether, these renal function abnormalities, with the exception of mild fluid retention, are clinically detectable in approximately 1% of exposed patients. Given the number of patients who take NSAIDs on a prescription or over-the-counter basis, the absolute number of at-risk patients is relatively large. Consequently, an appreciation for the risk factors and pathophysiology of NSAID-induced renal function abnormalities is required for optimal use of these drugs.*

Approximately 1–5 of persons who are exposed to a nonsteroidal anti-inflammatory drug (NSAID) will manifest one of a variety of renal function abnormalities. Although this percentage appears relatively low, the number of at-risk individuals is enormous because of the current use profile of NSAIDs, either as prescription or over-the-counter drugs. One in seven Americans is likely to be treated with an NSAID for a chronic rheumatologic disorder. If patients who take NSAIDs for acute problems are considered, the exposure rate will be even higher. Thus, of the 50 million Americans expected to use NSAIDs intermittently or routinely this year, at least 500,000 are likely to develop some degree of renal functional abnormality.

In descending order of frequency, the primary NSAID-related renal abnormalities are 1) fluid and electrolyte disturbances, 2) acute deterioration of renal function, 3) nephrotic syndrome with interstitial nephritis, and 4) papillary necrosis (Table I). Sodium chloride and water retention, the most commonly encountered renal effect of NSAID use, occurs to some degree in virtually all exposed persons but results in clinically detectable edema in less than 5% of patients. This rate is probably higher in selected at-risk patients. NSAID-induced fluid retention is typically benign, reversible on discontinuation of the NSAID, and easily managed in patients who require treatment. Other electrolyte abnormalities are also induced by NSAIDs, the most important of which is potassium retention and hyperkalemia. A high-risk group can also be identified for this electrolyte abnormality.

From the clinical point of view, the most worrisome renal side effect of NSAIDs is hemodynamically mediated acute renal failure, which occurs in individuals with pre-existing reduced renal blood perfusion. Ordinarily, the kidneys of such at-risk patients produce vasodilatory prostaglandins to maintain renal perfusion and function. The inhibitory effects of NSAIDs on renal prostaglandin production lead to acute, reversible renal failure in these patient. Acute deterioration of renal function occurs in 0.5 to 1% of patients who take NSAIDs on a chronic basis.

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**TABLE I**

**Documented Renal Effects of Nonsteroidal Anti-Inflammatory Drugs**

| Drug Class | Generic Name | Brand Name/ Manufacturer | Renal Effects* | | | | |
|---|---|---|---|---|---|---|---|
| | | | Edema | ↑K | ARF | NS | PN |
| Salicylates | Aspirin | (various) | CI | | CI | | CI |
| | Diflunisal | Dolobid/Merck | CI | | CI | CI | An |
| Propionic acids | Ibuprofen | Motrin/Upjohn | CI | CI | CI | CI | CI |
| | Naproxen | Naprosyn/Syntex | CI | | CI | CI | An |
| | Fenoprofen calcium | Nalfon/Lilly | CI | | CI | CI | CI |
| | Ketoprofen | Orudis/Wyeth-Ayerst | CI | | CI | CI | |
| | Flurbiprofen | Ansaid/Upjohn | CI | CI | CI | CI† | An |
| Indolacetic acids | Indomethacin | Indocin/Merck | CI | CI | CI | CI | CI‡ |
| | Sulindac | Clinoril/Merck | CI | CI | CI | CI | An |
| | Tolmetin | Tolectin/McNeil | CI | | CI | CI | An |
| | Diclofenac | Voltaren/Cibia-Geigy | CI | | CI | CI | CI |
| Anthranilic acids | Meclofenamate sodium | Meclomen/Parke-Davis | CI | | CI | CI | An |
| | Mefenamic acid | Ponstel/Parke-Davis | CI | | CI | CI | CI |
| Pyrazolones | Phenylbutazone | Butazolidin/Ciba-Geigy | CI | | CI | CI | CI |
| Oxicams | Piroxicam | Feldene/Pfizer | CI | CI | CI | CI | CI |

* *ARF = acute renal failure; NS = interstitial nephritis and nephrotic syndrome; PN = papillary necrosis; ↑K = hyperkalemia; CI = reported in clinical studies; An = described in studies in animals (but not in humans).*

† *Causes interstitial nephritis without nephrotic syndrome.*

‡ *Reported in combination with phenylbutazone.*

*(Adapted from Clive and Stoff,[1] with permission.)*

The nephrotic syndrome, with associated interstitial nephritis, is seen on rare occasions. Once again, it is reversible on discontinuation of the NSAID in question.

According to the respective manufacturers' prescribing information, chronic administration of nearly all NSAIDs produces papillary necrosis in laboratory animals; and a few clinical case reports of papillary necrosis can be found in the recent medical literature. Within the framework of our present understanding of NSAID effects on the kidney, this appears to be the only irreversible form of renal toxicity.

Many of the renal abnormalities that are encountered as a result of NSAID use can be attributed to the action of these drugs on prostaglandins. Hence, a brief overview of the interactions between prostaglandins and renal function will be presented, followed by an analysis of the pathophysiology, clinical manifestations, patient risk factors, and preventive approaches to NSAID-induced renal syndromes.

## THE PROSTAGLANDIN PATHWAY

Prostaglandins are ubiquitous substances that influence renal function along with a variety of other body systems.[1,2] Conceptually, they may be considered local hormones or "autocoids" because they act in a paracrine or autocrine fashion. Biologic activity is limited to the site of action by the short half-life of prostaglandins in circulation. In addition, prostaglandins are not stored in tissue, but are synthesized on demand.

Prostaglandins are derived from phospholipids by a common pathway (Figure 1). Phospholipids, of course, are widely distributed in cell membranes throughout the body. The most important precursor for prostaglandins is arachidonic acid. Cyclooxygenase is the catalyst for oxygenation of arachidonic acid, which is the step that is inhibited by NSAIDs. The interaction between aspirin and cyclooxygenase (acetylation) is irreversible, whereas that with other NSAIDs is reversible.

Arachidonic acid can also be metabolized to other mediators, depending on the cell type. For example, lipoxygenase catalyzes the production of leukotrienes, and mixed-function oxygenases catalyze the production of epoxyeicosatrienoic acids. Collectively, these oxygenated metabolites of arachidonic acid are known as eicosanoids because of their origin from a 20-carbon (eicosa-) polyunsaturated acid.[3]

Continuing along the common pathway (Figure 1), oxygenation of arachidonic acid results in production of prostaglandin $G_2$, which is converted to prostaglandin $H_2$ by hydroperoxidase and loss of a free radical. At this point, metabolism becomes highly specific for individual cell types, although many, if not all, of the metabolites are produced in the kidney. Prostaglandin $E_2$ is a vasodilator, which, in the kidney, promotes diuresis and natriuresis. Prostaglan-

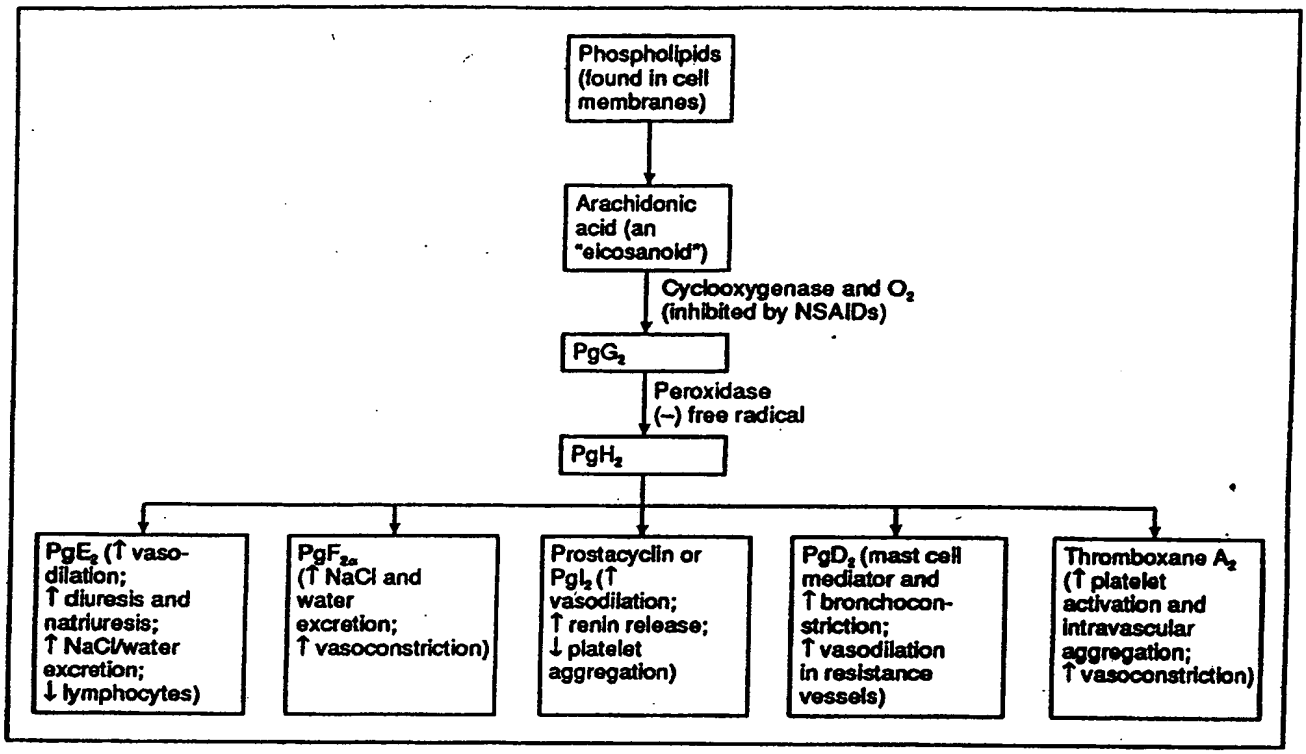


*Figure 1. Prostaglandin pathway (and prostanoid functions). Pg = prostaglandin; ↑ = stimulate or increase; ↓ = inhibit or decrease.*[2,3]

din $E_2$ also inhibits lymphocytes and other cells that are involved in inflammation and allergic responses, which, as will be discussed later, may play a role in some NSAID-induced renal syndromes. Prostaglandin $F_{2\alpha}$ enhances excretion of sodium chloride and water. Prostacyclin, also known as prostaglandin $I_2$, has a wide variety of actions including vasodilation, renin release, and inhibition of platelet aggregation. Prostaglandin $D_2$ is a vasodilator of peripheral resistance vessels but is better known for its association with mast cell activation and bronchoconstriction. Thromboxane $A_2$ is the principal metabolite of prostaglandin $H_2$ in platelets and can act as a major vasoconstrictor within the kidney. These pharmacologically active metabolites of prostaglandin $H_2$ are collectively known as prostanoids.[3]

## PROSTAGLANDIN EFFECTS ON RENAL FUNCTION

Given the diversity of cell populations within the kidney and their various functions, the complexity of the interactions between prostaglandins and renal function is not unexpected. Prostaglandins are involved in renin release, local vascular tone, regional circulation, sodium and water homeostasis, and potassium balance (Table II). The following sections describe these diverse effects. Detailed overviews of these interactions can be found in excellent reviews by Patrono and Dunn[2] and Oates and colleagues.[3]

An important caveat in the following sections is that prostaglandins are not primary mediators of basal renal function in normal individuals. Prostaglandins typically operate in conjunction with a variety of other mediators, which, even in the absence of prostaglandins, can preserve homeostasis. Prostaglandin production is increased as needed in response to stress (e.g., decreased renal blood flow or blood volume). Thus, inhibition of prostaglandin function by NSAIDS is more likely to cause complications in at-risk patients with decreased renal blood perfusion than in the otherwise normal subject whose prostaglandins are merely one of many factors contributing to homeostasis.

### Renin Release

Prostaglandins stimulate renin release, which plays an important role in the regulation of arterial blood

**TABLE II**

**Principal Renal Sites of Prostaglandin Synthesis and Major Actions**

| Site | Eicosanoid | Action |
|---|---|---|
| Vasculature | Prostaglandins $I_2$ and $D_2$ | Vasodilation |
| Glomerulus | Prostaglandins $I_2$ and $E_2$ | Maintain GFR |
| | Thromboxane $A_2$ | Reduce GFR |
| Collecting tubule | Prostaglandins $E_2$ and $F_{2\alpha}$ | Enhance excretion of sodium chloride and water |
| Medullary interstitial cells | Prostaglandin $E_2$ | Vasodilation and natriuresis–diuresis |

*(Adapted from Patrono and Dunn[3], with permission.)*

pressure, blood volume, and electrolyte balance. Prostaglandins can act independently or synergistically with the β-adrenergic system.[4] Although the exact prostanoid mediator is not yet known, it is likely that prostacyclin is synthesized in response to a change in arteriole pressure or chloride reabsorption in the macula densa of the nephron.[3]

### Local Vascular Tone

Prostanoids are one of several local mediators that govern vascular tone through their actions on norepinephrine release at peripheral nerve endings. Prostaglandins $E_2$ and $D_2$ and, to a lesser extent, prostacyclin promote vasodilation by inhibiting norepinephrine release. Prostaglandin $E_2$ also antagonizes the effects of angiotensin II, a powerful vasopressor, on the neuroeffector junction. Conversely, prostaglandin $F_{2\alpha}$ and thromboxane $A_2$ are vasoconstrictors.[3]

### Regional Circulation

Prostanoids contribute to regional circulation via their influence on local vascular tone. Under normal conditions, prostanoids do not regulate renal perfusion per se. However, certain conditions such as decreased renal blood flow are associated with the production of vasodilatory prostaglandins. Prostaglandin $E_2$, prostacyclin, and prostaglandin $D_2$ shift regional blood flow from cortical to juxtamedullary nephrons.[3]

### Sodium and Water Homeostasis

All prostanoids are capable of acting in the renal cortex to regulate sodium and water homeostasis; however, prostanoids are only one of many factors that share this function.[3] Prostaglandins $E_2$ and $D_2$, prostacyclin, and, to a lesser extent, prostaglandin $F_{2\alpha}$ increase the rate of salt and water excretion. Prostaglandin $E_2$ inhibits sodium chloride transport in the thick ascending limb of the loop of Henle and the collecting duct.[5,6] In addition, prostaglandins antagonize the effects of antidiuretic hormone.[7,8]

Prostanoids do not have a direct effect on glomerular filtration rate; however, vasodilation associated with prostaglandin $E_2$, prostacyclin, and prostaglandin $D_2$ increases renal blood flow, and, as previously mentioned, shunts blood flow from the cortical to juxtamedullary nephrons. The net result is enhanced diuresis and natriuresis due to reduced medullary hypertonicity and increased interstitial pressure.[3]

### Potassium Balance

Prostanoids indirectly lower potassium by their effects on glomerular filtration and renin.[3] As previously mentioned, vasodilatory prostaglandins increase renal blood flow. This may enhance the direct intratubular delivery of potassium into the distal nephron for excretion. Alternatively, this may serve to quantitatively increase sodium delivery into the distal nephron with resultant reabsorption of sodium in exchange for potassium, which is then excreted in the urine. Secondly, prostacyclin is believed to promote renin release. Activation of the renin-angiotensin pathway ultimately causes aldosterone to stimulate potassium excretion in the distal convoluted tubule and collecting duct. However, potassium balance is also regulated by a number of other factors such as insulin and the β-adrenergic system.

## FLUID AND ELECTROLYTE DISTURBANCES

### Sodium and Water Retention

The most common and universal renal complications of NSAIDs are sodium retention and edema. According to prescribing information accompanying nearly all NSAIDs, edema occurs in at least 3% of patients. The incidence is probably higher in patients who take therapeutic doses over prolonged periods. The onset of fluid retention usually occurs early in the course of therapy and can be dramatic as

illustrated by the 15-kg weight gain in a 70-year-old man who took ibuprofen for only 17 days.[9]

Occasionally, the patient may retain water in excess of sodium. Severe, reversible hyponatremia (118 μmol $Na^+$/L) occurred in a patient who took ibuprofen for only 3 days. This patient had underlying renal impairment (CrCl 12 mL/min).[10]

The multiple mechanisms by which NSAIDs interfere with water and sodium metabolism may explain the frequency of this complication. As previously mentioned, NSAIDs have the potential to disrupt diuresis and natriuresis by interfering with prostaglandin-mediated sodium chloride transport, antidiuretic hormone, and distribution of blood flow from cortical to juxtamedullary nephrons.[1,3] The hypothesis for the pathogenesis of the nephrotic syndrome is also operative in this situation. By shunting arachidonic acid metabolism from prostaglandins to lipoxygenase products, NSAIDs may favor production of eicosanoid derivatives that increase capillary permeability.[1]

### Hyperkalemia

Hyperkalemia is an unusual complication of NSAIDs, presumably because of the multiplicity of factors that are capable of maintaining potassium balance, even in the absence of prostaglandins. Hyperkalemia is more likely to occur in patients with preexisting renal impairment,[11,12] cardiac failure,[13] diabetes,[12] or multiple myeloma[14] or in patients who receive potassium supplementation,[15] potassium-sparing diuretics,[16] or angiotensin-converting enzyme (ACE) inhibitors. Indomethacin appears to be the major NSAID associated with this complication and has produced hyperkalemia in patients without apparent risk factors.[17] Thus, indomethacin may have a direct effect on the cellular uptake of potassium,[18] in addition to the known effects of NSAIDs on potassium delivery to the distal tubule as well as on the renin-angiotensin and aldosterone pathways.

NSAID-induced hyperkalemia often occurs in the setting of NSAID-induced acute renal deterioration or worsening of underlying renal impairment. However, the severity of hyperkalemia can be disproportionate to that of renal impairment. For example, Tan and colleagues reported a patient who was treated with indomethacin and had a serum potassium of 6.2 mEq/L in spite of only mildly abnormal renal function.[19] In this patient, plasma renin and aldosterone levels were suppressed and did not respond to furosemide or postural changes. Urinary prostaglandin $E_2$ was also suppressed. Discontinuation of indomethacin resulted in normalization of potassium, prostaglandin $E_2$, and a rebound of renin and aldosterone.

## ACUTE DETERIORATION OF RENAL FUNCTION

### Role of Prostanoids in Maintaining Renal Blood Flow

Although NSAIDs do not impair glomerular filtration in normal individuals,[20,21] acute renal decompensation may occur in at-risk patients with various extrarenal or renal disease processes that lead to decreased renal perfusion (Table III). Renal prostaglandins play an important role in the maintenance of homeostasis in these patients, so drug-induced disruption of counter-regulatory mechanisms can produce clinically important and even severe renal functional deterioration.[2,3]

Acute renal deterioration in this setting can be attributed to the interruption of the delicate balance between hormonally mediated pressor mechanisms and prostaglandin-related vasodilatory effects (Figure 2). In at-risk patients, volume contraction triggers pressor responses via adrenergic and renin-angiotensin pathways. Ordinarily, vasodilatory renal prostaglandins counterbalance the vasoconstrictive effects of norepinephrine and angiotensin II. The addition of NSAIDs increases the risk of azotemia and possibly ischemic damage to the kidney by removing the protective effects of vasodilatory prostaglandins and allowing unopposed vasoconstriction.

### Clinical Features of Acute Renal Failure

Initially, this NSAID-induced renal syndrome is of moderate severity and is characterized by increasing BUN, creatinine, potassium, and weight with decreasing urine output. NSAID-induced acute renal failure is usually reversible over 2 to 7 days after discontinuation of therapy; however, morbid consequences can occur if the diagnosis is not recognized early. Continued NSAID therapy in the setting of de-

**TABLE III**

**At-Risk Patients for NSAID-Induced Acute Renal Failure**

| |
|---|
| Severe heart disease (congestive heart failure) |
| Severe liver disease (cirrhosis) |
| Nephrotic syndrome (chronic renal disease) |
| Elderly population |
| Dehydration (protracted) |

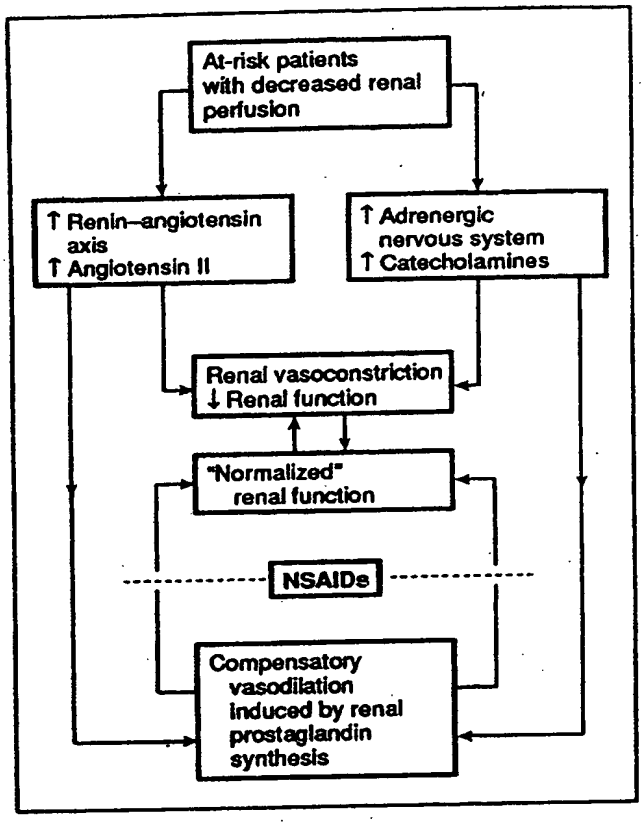


*Figure 2. Mechanism by which NSAIDs disrupt the compensatory vasodilation response of renal prostaglandins to vasoconstrictor hormones in patients with prerenal conditions. A solid line (—) indicates stimulation; a dashed line (---) indicates inhibition.*

teriorating renal function may progress rapidly to the point wherein dialysis support is required.[22] Despite this profound level of renal functional impairment, the kidney will nonetheless recover several days to weeks after discontinuation of the NSAID. Development of this type of "total" renal failure, which is often inappropriately designated as "acute tubular necrosis," represents the extreme end of the spectrum of hemodynamic insult rather than a separate clinical entity.

## Risk Factors for Acute Renal Failure

The risk of acute renal deterioration is highest in patients with liver disease, pre-existing renal impairment, cardiac failure, protracted volume contraction due to diuretic therapy or intercurrent disease, or old age. NSAID-induce renal decompensation has been well documented in patients with cirrhosis, particularly when ascites is present.[3] Urinary excretion of prostaglandin $E_2$, prostacyclin metabolites, and thromboxane $A_2$ is increased in these patients.[23,24] An analogous situation exists in patients with underlying congestive heart failure,[25] nephrotic syndrome,[26,27] or lupus nephritis.[28,29]

Patients with chronic renal impairment are at increased risk of NSAID-induced renal failure because of inadequate renal prostaglandin production. We documented NSAID-induced acute renal failure in patients with asymptomatic mild, but chronic, renal failure (serum creatinine between 1.5 and 3.0 mg/dL).[30] Baseline excretion of urinary prostaglandin $E_2$ and 6-keto-prostaglandin $F_{1\alpha}$ was quantitatively lower in the individuals who developed NSAID-induced renal decompensation than in those who did not, and ibuprofen proved to be more problematic than either piroxicam or sulindac. On initiation of ibuprofen, urinary prostaglandin excretion fell in all patients, but trough concentrations were quantitatively lower in the subset of patients who experienced acute renal failure.

Volume contraction due to diuretic therapy or an intercurrent disease that results in dehydration represents another important risk factor for the development of NSAID-induced acute deterioration of renal function.[22,31,32] Elderly patients are also at increased risk. We estimate that age of 80 years or greater is an independent risk factor because the physiology of ageing within the kidney results in 50% loss of function in 50% of the population at age 80, primarily as a result of the progression of arteriolonephrosclerosis.

## Pharmacodynamics of Acute Renal Failure

NSAID-induced acute renal decompensation is a pharmacologically predictable phenomenon that occurs in a dose-related fashion. In our triple-crossover study of 12 women with mild renal failure, ibuprofen (800 mg three times daily) was discontinued on day 8 because of worsening renal function (≥1.5 mg/dL increase in serum creatinine) or hyperkalemia (potassium ≥ 6 mEq/mL) in 3 patients. When these patients were rechallenged at a 50% lower dose of ibuprofen, two patients again had evidence of acute renal deterioration.[30]

Another important finding in our study was the time of onset of acute renal decompensation.[30] Ibuprofen-induced renal failure occurred rapidly (within days), but piroxicam and sulindac did not cause renal deterioration during the 11-day treatment period. A pharmacokinetic analysis in these patients provides insight. Ibuprofen, which has a short elimination half-life, reached maximum serum

concentrations quickly. In contrast, piroxicam and sulindac have longer half-lives and continued to accumulate throughout the treatment period. These findings are consistent with basic pharmacologic principles and suggest that NSAIDs having short elimination half-lives will reach steady state and exert maximum pharmacologic effects before NSAIDs having longer half-lives.

### "Renal Sparing" NSAIDs — ?

Although all NSAIDs have the potential to induce acute renal impairment, some quantitative differences may exist. Sulindac has been hypothesized to be renal sparing, possibly because of its unusual metabolic pathway.[33] The parent compound, sulindac sulfoxide, is an inactive prodrug that undergoes hepatic metabolism to sulindac sulfide, which is the metabolite that exerts anti-inflammatory activity. Sulindac sulfoxide is also metabolized to a much lesser extent to an inactive metabolite, sulindac sulfone. It has been hypothesized that, within the kidney, sulindac sulfide is reversibly oxidized to the inactive parent compound, sulindac sulfoxide, such that renal prostaglandin production would not be influenced.

In clinical studies, urinary prostaglandin levels and renal effects were unchanged in patients with normal renal function[34,35] and states of proteinuria.[36] However, the duration of sulindac in these studies may have been insufficient to appreciate the full pharmacologic effect of sulindac. NSAID-induced changes may not have been detectable because of the presence of only very mild renal impairment or absence of renal failure altogether in these studies. Longer courses of sulindac in patients with slightly more severe renal impairment have been associated with statistically significant reductions in urinary prostaglandins[30] and glomerular filtration rate.[37]

The ability of sulindac to inhibit prostaglandin synthesis and impair renal function has been confirmed in a different high-risk group, namely patients with hepatic cirrhosis and ascites.[38] We have also identified the development of profound acute renal failure in high-risk patients who received sulindac for several days to weeks. Collectively, these clinical experiences indicate the need for cautious and timely monitoring of high-risk patients who receive NSAIDs.

## NEPHROTIC SYNDROME WITH INTERSTITIAL NEPHRITIS

NSAIDs also cause another type of renal dysfunction that is associated with various levels of functional impairment and characterized by the development of the nephrotic syndrome with interstitial nephritis.[1,22,39,40] The clinical features, absence of risk factors, and pathophysiology distinguish this from other NSAID-induced renal syndromes and from classic drug-induced allergic interstitial nephritis.

The features of this NSAID-induced renal syndrome are variable. The patient may experience edema, oliguria, and/or foamy urine.[41] Systemic signs of allergic interstitial nephritis such as fever, drug rash, peripheral eosinophilia, and eosinophiluria are generally absent.[1,22,40,41] The urine sediment contains microscopic hematuria and pyuria.[1,41] Proteinuria typically is in the nephrotic range.[1,39] We have noted that renal functional deterioration can range from minimal to severe.

Characteristically, this form of nephrotic syndrome consists of minimal change glomerulonephritis with interstitial nephritis, which is an unusual combination of histologic findings. NSAID-induced nephrotic syndrome without interstitial disease is rare but has been reported in a handful of patients who took fenoprofen, sulindac, or diclofenac. Conversely, interstitial disease without nephrosis has been reported in a few patients, but this may, in fact, represent allergic interstitial nephritis.[41]

In spite of nephrotic-range proteinuria, the most impressive histopathologic findings involve the interstitium and tubules. A focal diffuse inflammatory infiltrate can be found around the proximal and distal tubules. We reported that the infiltrate primarily consisted of cytotoxic T lymphocytes but also contained other T cells, B cells, and plasma cells.[39] Changes in the glomeruli were minimal and resembled those of minimal change glomerulonephritis with marked epithelial-foot process fusion. Other investigators have reported similar findings.[1,22,41,42]

The onset of NSAID-induced nephrotic syndrome is usually delayed, having a mean time of onset of 5.4 months after initiation of NSAID therapy[40] and ranging from 2 weeks to 18 months.[1] NSAID-induced nephrotic syndrome is usually reversible 1 month to 1 year after discontinuation of NSAID therapy. During the recovery period, some patients may require dialysis. Corticosteroids have been used empirically, but it is not clear whether they hasten recovery.[1,22,39] If proteinuria does not significantly remit within 2 weeks after discontinuation of the NSAID, we recommend a standard, 2-month trial of corticosteroid therapy as would be employed in a nephrotic adult with idiopathic minimal change or membranous glomerulonephritis.

Risk factors are not well understood. Underlying renal impairment does not appear to be a risk factor. Old age has been suggested as a risk factor,[22,40] but

this may also be a reflection of the usual candidate for chronic NSAID therapy. The syndrome has been more commonly reported with fenoprofen than other NSAIDs. Approximately two-thirds of cases have been associated with fenoprofen. Hence, the structure of the drug itself appears to be of major importance. The syndrome has been attributed, nonetheless, to virtually all NSAIDs, including those from structurally distinct classes.[1,22,39,40,41]

The mechanism of NSAID-induced nephrotic syndrome has not been fully characterized. The association of this syndrome with structurally distinct NSAIDs suggests a common denominator. T lymphocytes may function as immune mediators instead of the humoral factors that are responsible for classic drug-induced allergic interstitial nephritis. In keeping with this hypothesis, NSAID-induced prostaglandin inhibition may play an indirect role. By inhibiting cyclooxygenase, NSAIDs may promote metabolism of arachidonic acid to non-prostaglandin eicosanoids. Indeed, leukotrienes, the products of the interaction between lipoxygenase and arachidonic acid, are known to recruit T lymphocytes and promote the inflammatory process. Leukotrienes may also contribute to proteinuria by increasing vascular permeability.[1,40,41]

## PAPILLARY NECROSIS

Papillary necrosis with interstitial nephritis is a well-known complication of chronic phenacetin abuse that has been reviewed extensively elsewhere.[43] Fortunately, the incidence of the latter complication has diminished considerably because of a better understanding of the pathophysiology and patient education. It has been suggested that chronic aspirin alone may also induce papillary necrosis,[44] but it is not clear that this can actually occur. What is clinically apparent is that chronic (10 to 20 years) exposure of the kidney to high doses of analgesic combinations such as salicylate and acetaminophen (the metabolite of phenacetin), often with the addition of caffeine, can and will produce chronic, progressive papillary necrosis.

The black pigmentation found within necrotic papillae associated with phenacetin abuse (or phenacetin-containing combinations) is absent in patients who ingest aspirin alone or other NSAIDs. This black pigmentation may represent a breakdown product of phenacetin.[43]

In preclinical studies, nearly all of the NSAIDs produced papillary necrosis in experimental animal models. Clinical toxicity is exceedingly rare but has been reported for ibuprofen,[45] phenylbutazone,[46,47] fenoprofen,[48] and mefenamic acid,[49] and according to prescribing information, several other NSAIDs.

The typical candidate for NSAID-induced papillary necrosis is a middle-aged woman with a history of ingesting over-the-counter, combination analgesics for headache. Closer questioning may reveal that the patient takes the analgesic for the mood-altering effects of caffeine. Renal manifestations may include loin pain, macroscopic hematuria, ureteral obstruction, and/or uremia. Urinary tract infection and hypertension are common secondary findings. Reversibility is determined by the extent of deterioration and ability to discontinue NSAID therapy.[43] Recent reports from the FDA[50] of spontaneous gross hematuria associated with NSAIDs such as ibuprofen (three cases) suggest that papillary necrosis also occurs with newer NSAIDs. These data suggest a minor degree of papillary damage, but chronic progressive deterioration of renal function is not a feature of most reports.

The mechanism of NSAID-induced papillary necrosis is not clear. The causative role of NSAIDs is difficult to delineate because of the presence of confounding factors such as underlying disease, urinary tract infection, and/or concomitant medications. Selected NSAIDs may exert a direct toxic effect on renal papillae, particularly combinations of aspirin and acetaminophen, a major metabolite of phenacetin. Both drugs are highly concentrated in the medulla. Aspirin depletes cellular glutathione, which would otherwise neutralize the acetaminophen metabolite, N-acetyl-benzo-quinoneimine. Without glutathione, this highly reactive metabolite could lead to cell death.[43]

Prostaglandin inhibition may also play a role.[1] Medullary ischemia, a possible precipitating factor in development of papillary necrosis, results from NSAID-induced reduction in blood flow to the renal medulla in experimental models.[51,52]

## OTHER NSAID-INDUCED RENAL SYNDROMES

Phenylbutazone, suprofen, and benoxaprofen produce unique renal syndromes that are of historic interest. These complications are rarely encountered because phenylbutazone use has diminished because of the availability of safer drugs, and suprofen and benoxaprofen have been removed from the market.

Two mechanisms have been identified for phenylbutazone-induced acute oligo-anuric renal failure.[1] Phenylbutazone is known to inhibit uric acid reabsorption, which may cause hyperuricosuria, and ultimately, bilateral ureteral obstruction due to uric acid stones.[53] Secondly, an idiosyncratic reaction has

been reported that results in acute tubular injury without uric acid precipitation.[54] Underlying renal impairment is a risk factor for the latter reaction. Also, patients experiencing this reaction appear to be predisposed to subsequent renal injury from other NSAIDs. These observations suggest that prostaglandin inhibition may play a role in the development of the idiosyncratic reaction.[1]

Suprofen-induced acute renal failure is characterized by acute flank and/or abdominal pain, occurring within 12 hours after starting therapy. In a series of 16 patients described by Hart and colleagues,[55] the mean peak serum creatinine was 3.6 mg/dL (range: 2–8 mg/dL) and was within normal limits at follow-up in most patients. Urinalysis revealed microhematuria (8/12 patients) and proteinuria (7/12 patients) but no crystals. One of our patients with suprofen-induced flank pain syndrome had birefringent crystals in the urine several hours after the injection of the drug and at a time when rehydration had already been commenced. We did not determine if these crystals were uric acid or drug metabolites.

The mechanism of suprofen-induced flank pain and acute renal failure was never conclusively identified before the drug was removed from the market. No obvious risk factors were identified in the previous series since all patients appeared to be in good health and took NSAIDs for acute symptomatic relief. It has been hypothesized that the suprofen flank pain syndrome is related to acute uric acid crystal precipitation within the nephron leading to acute urinary flow obstruction.[50,55] Suprofen is known to have uricosuric activity. The finding of hyperuricemia (mean: 10.8 mg/dL) in four of four patients suggests that this may be a risk factor.[55]

Benoxaprofen, an NSAID with a long half-life, was removed from the market in 1982, within weeks after its introduction, because of adverse effects. It is remembered for severe hepatic toxicity that occasionally resulted in death; however, renal failure was also a contributing factor. Risk factors for benoxaprofen-induced toxicity were old age and concomitant diuretic therapy, two factors known to increase the risk of acute functional renal failure.

## CONCLUSIONS

NSAIDs are considered safe and suitable for the treatment of a variety of chronic and acute conditions. The risk of renal failure after the initiation of any given NSAID is low; however, the number of at-risk patients is high because of the widespread use of these drugs.

In most cases, NSAID-induced renal syndromes are a direct or indirect result of prostaglandin inhibition, which has important clinical implications. At this time, it is not clear whether it is possible to completely separate the effects of NSAIDs on systemic prostaglandins, which mediate anti-inflammation activity, from renal effects. Thus, under the right cir-

**TABLE IV**

**Summary of Effects of NSAIDs on Renal Function**

| Renal Syndrome | Mechanism | Risk Factors | Prevention/Treatment |
|---|---|---|---|
| Sodium retention and edema | ↓ Prostaglandin | NSAID therapy (most common adverse effect) | Stop NSAID |
| Hyperkalemia | ↓ Prostaglandin, ↓ potassium to distal tubule and ↓ aldosterone/renin–angiotensin | Renal disease<br>Heart failure<br>Diabetes<br>Multiple myeloma<br>Potassium therapy<br>$K^+$-sparing diuretic | Stop NSAID<br>Avoid indomethacin in high-risk patients |
| Acute deterioration of renal function | ↓ Prostaglandin and disruption of hemodynamic balance | Liver disease<br>Renal disease<br>Heart failure<br>Dehydration<br>Old age | Stop NSAID<br>Avoid use in high-risk patients |
| Nephrotic syndrome with interstitial nephritis | ↑ Lymphocyte recruitment and activation | Fenoprofen | Stop NSAID<br>Dialysis and (?) steroids as needed |
| Papillary necrosis | Direct toxicity | Phenacetin abuse<br>Aspirin–acetaminophen combination | Stop NSAID<br>Avoid chronic analgesic use |

cumstances, virtually any NSAID can produce renal complications. Fortunately, these complications are usually reversible if the diagnosis is recognized promptly and NSAID therapy is discontinued.

With an understanding of the pathophysiology involved, preventive clinical measures can be put into operation. Risk factors have been identified for most NSAID-induced renal syndromes (Table IV). It is prudent to avoid high-dose, chronic NSAID therapy in at-risk patients (Table III). Unfortunately, this is not always possible. If NSAIDs are necessary in these high-risk groups, the patients should be monitored closely and receive appropriate counselling. Monitoring should begin within a week after initiation of a short-acting NSAID (e.g., ibuprofen) and continue indefinitely for signs of syndromes having delayed onset (e.g., nephrotic syndrome with interstitial nephritis).

In the event of NSAID-induced renal failure, the NSAID should be discontinued promptly. The patient should receive supportive care as needed. After stabilization of renal function, rechallenge with the same dose of the offending drug or even a structurally unrelated NSAID is likely to reproduce the adverse effect. (Patients who have recovered from an episode of protracted dehydration due to diuretics or intercurrent disease are an exception to this rule.) Thus, if anti-inflammatory therapy is mandatory, underlying risk factors should be identified and eliminated, if possible. Unfortunately, this is often not possible, as in the case of old age or chronic heart, kidney, or liver disease. These patients may require alternative therapy using corticosteroids or other supportive drugs such as acetaminophen or colchicine.

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# The Renal Effects of Nonsteroidal Anti-inflammatory Drugs: Summary and Recommendations

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● The renal effects of nonsteroidal anti-inflammatory drugs are reviewed with special emphasis on the clinical, pathophysiologic, and risk factors for acute renal failure. Renal papillary necrosis and chronic renal insufficiency can occur with the prolonged use of these drugs, although the prevalence of this manifestation of nonsteroidal anti-inflammatory drug nephrotoxicity is unknown. Current recommendations based on a critical literature survey are provided, along with a list of suggested areas in which more research is needed.


INDEX WORDS: Nonsteroidal anti-inflammatory drugs; acute renal failure; chronic renal failure; analgesic nephropathy; prostaglandins.

NONSTEROIDAL anti-inflammatory drugs (NSAIDs) are popular and used widely because of their acknowledged efficacy and excellent safety profile in a wide range of clinical conditions. Despite their many useful therapeutic applications, there is now substantial evidence arising from experimental studies and clinical studies in humans for multiple effects of NSAIDs on kidney function. This is not surprising since the principal action of NSAIDs is to block the synthesis of cyclo-oxygenase products of arachidonic acid, which have a critical modulatory role on renal hemodynamics, renal epithelial cell fluid and ion transport, and the synthesis and action of renal hormones. Nonsteroidal anti-inflammatory drugs are now available both in over-the-counter and prescription strengths. The majority of healthy, normal subjects who ingest therapeutic dosages of NSAIDs for limited duration tolerate these drugs without adverse effects. However, a subset of individual are susceptible to subclinical as well as serious renal toxicity from these agents. In addition to the effects listed in Table 1, NSAIDs interfere with the efficacy of antihypertensive medicines, leading to an increase in blood pressure.

Since the toxicity of NSAIDs in the kidney is linked to the disruption of renal prostaglandin synthesis, a brief review of the renal effects of prostaglandins and the consequences of synthesis interruption is in order.

## PROSTAGLANDIN SYNTHESIS AND COMPARTMENTALIZATION

Prostaglandins are derivatives of arachidonic acid, a 20-carbon tetraenoic acid, which is acylated to membrane phospholipids. Deacylation of arachidonic acid from the cell membrane is controlled by phospholipases, predominantly phospholipase $A_2$. Vasopressin,[1] bradykinin,[2] angiotensin,[3] and norepinephrine[4] all stimulate arachidonic acid release from membranes, whereas glucocorticoids inhibit release.[5] After arachidonic acid is released from the cell membrane, several synthetic pathways are then available. Molecular oxygen may be added to the arachidonic acid by the action of an intracellular endoplasmic reticulum-bound peroxidase enzyme (cyclo-oxygenase), which leads to the synthesis of endoperoxide $PGG_2$. A second endoperoxide ($PGH_2$) is then formed with the liberation of a superoxide radical. Once formed, $PGH_2$ has a short half-life and is rapidly acted on by a series of enzymes that produce the biologically active molecules. Nonsteroidal anti-inflammatory drugs exert their prostaglandin inhibitory effects by primarily inhibiting the activity of cyclo-oxygenase by 70% to 95%. Prostaglandin biosynthesis is also decreased by NSAIDs, reducing the generation of superoxide and hydroxyl-free radicals.[6,7]

The endoperoxide $PGH_2$ is transformed by a series of enzymes to the dienoic series of prostaglandins. These prostaglandin metabolites possess biologic activity in the kidney; for example, prostacyclin synthetase acts to form prostacyclin ($PGI_2$), whereas thromboxane synthetase forms thromboxane ($TXA_2$) and the isomerases act to

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Table 1. Kidney Manifestations of NSAIDs

| Kidney Toxicity | Mechanism | Risk Factors |
|---|---|---|
| Acute renal failure | Loss of counterregulatory prostaglandins | Plasma volume contraction, congestive heart failure, cirrhosis, and ascites |
| Sodium retention | Loss of natriuretic prostaglandins | Unknown |
| Potassium retention | Hyporeninemic hypoaldosteronism | Concomitant defects in potassium homeostasis |
| Water retention | Enhanced antidiuretic hormone action, increased medullary tonicity | Unknown |
| Acute interstitial nephritis | Reactive arachidonic acid metabolite | Unknown |

form $PGE_2$ and $PGF_2$. Prostaglandins are known to exert physiologic effects at the locations at which they are synthesized. In this regard, they are really autocoids rather than true hormones. Prostaglandins that are excreted into renal lymph or into the renal vein are rapidly metabolized into active products in the lung. The prostaglandin synthetic pathway is shown in Fig 1. Prostaglandins synthesized in the renal cortex regulate renal cortical processes (renal vascular resistance and renal secretion), whereas prostaglandins formed in the medulla modulate medullary physiologic events (salt and water handling). The most abundant prostaglandin found in the tubules is $PGE_2$. The cortical and particularly medullary portion of the collecting duct are the dominant sites of $PGE_2$ synthesis. Medullary interstitial cells are also a rich source of $PGE_2$ production. Prostaglandin $E_2$ undergoes spontaneous hydrolysis to 6-keto-$PGF_{1\alpha}$. Prostaglandins are rapidly metabolized into inactive products by a 15-prostaglandin dehydrogenase.

## EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON RENAL FUNCTION: CLINICAL CONSEQUENCES

Under baseline and euvolemic circumstances there is typically a very low rate of prostaglandin synthesis. Because this is true in a healthy state, it is difficult to demonstrate that prostaglandins contribute to the normal maintenance of renal function even when using powerful cyclo-oxygenase inhibitors, such as NSAIDs. When prostaglandin synthesis is upregulated as hemodynamic destabilization occurs, the synthesis and release of prostaglandins is greatly enhanced. Under these circumstances the inhibition of prostaglandin synthesis has been clearly demonstrated to have profound adverse hemodynamic effects on the kidney. Most of these effects have been seen

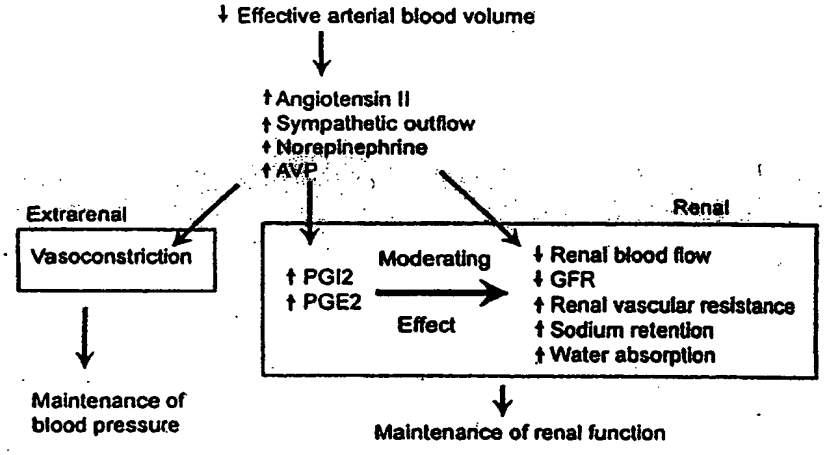


Fig 1. Pathways for prostaglandin formation. (Reprinted with permission.[25])

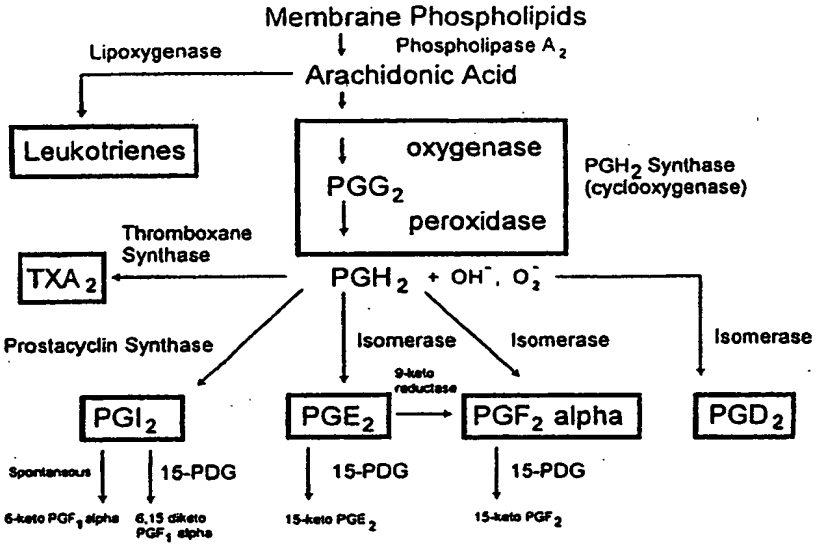


**Fig 2. Schematic depiction of the relationship between vasodilator and vasoconstrictor input into the kidney. $PGI_2$ and $PGE_2$ exert a moderating effect on renal vasoconstrictive stimuli.**

in circumstances in which blood volume or effective arterial blood volume is compromised and vasoconstrictor peptide secretion would be expected to be high. Angiotensin II, norepinephrine, vasopressin, and sympathic nerve activity all increase under these perturbed circumstances and cause an increase in renal vascular resistance. In addition, each of these stimuli is a potent agonist for prostaglandin synthesis.[2,3,8] Hence, what ensues is a dynamic interplay between counterbalancing vasoconstrictor and vasodilator forces. It is under these circumstances that the inhibitor of prostaglandin synthesis will result in excessive vasoconstriction, with a consequent decrease in renal blood flow and finally a decrement in glomerular filtration rate. These relationships are graphically depicted in Fig 2.

## ACUTE RENAL FAILURE

Acute renal failure (ARF) due to a decrease in renal blood flow secondary to increase renal vascular resistance has been well described. The afferent renal arteriole appears to be under tonic regulation by vasodilator prostaglandins, and loss of these dilators leads to vasoconstriction and a decrease in glomerular capillary pressure, resulting in a prompt decline in glomerular filtration rate. This form of renal failure is often sudden, presenting with oliguria and a decrease in fractional sodium excretion. Withdrawal of NSAIDs usually leads to prompt reversal of the ARF. Virtually all NSAIDs have been implicated, although some subclasses of NSAIDs may be less toxic because of renal conversion of active drug to inactive metabolite.

A common risk factor of ARF is the physiologic state of plasma volume depletion induced either by hemorrhage, salt loss, or hypoalbuminemia. In these conditions, circulating vasoconstrictors are released, maintaining vascular resistance and blood pressure at the potential expense of regional organ blood flow. To maintain blood flow, particularly in the kidney, counterregulatory renal prostaglandins are released that counteract vasoconstrictors and normalize renal blood flow. Nonsteroidal anti-inflammatory drugs taken under these circumstances blunt this counterregulatory response and intensify the renal vasoconstriction leading to ARF. If the vasoconstriction is sufficiently intense and of extended duration, acute tubular necrosis may ensue. Similar physiology to intravascular volume depletion is seen in severe congestive heart failure (New York Heart Association grade III, IV) and hepatic failure with ascites. In these two pathophysiologic states, which are also associated with activation of circulating neurohumoral vasoconstrictors, NSAID use may lead to ARF by augmenting arteriolar constriction.

## INTERRUPTION OF RENAL TUBULAR ION AND WATER TRANSPORT: CLINICAL CONSEQUENCES

Eicosanoids or oxygenated metabolites of arachidonic acid exert modulatory influences on many ion transport sites along the nephron. Consequently, their synthesis interrupted by NSAID use leads to a wide variety of disorders of ion transport. Most prominent among these in clinical use is the retention of sodium. Virtually all individuals will develop positive sodium retention following the use of NSAIDs and escape from this antinatriuretic effect in several days. A small subset of individuals fail to escape and develop a severe edema state. Natriuresis rapidly ensues once the drug is discontinued.

An issue related to sodium retention is the effect of NSAIDs to antagonize the effect of concomitant diuretic use. This antagonism has been described for the use of both thiazide and loop diuretics. Potassium-sparing diuretics, particularly triamterene, have been implicated as a potential risk factor for NSAID-induced ARF. Reports of this combination of drugs have been in the form of case reports and require further study to document the precise risk.

Hyperkalemia is the second major electrolyte disorder that accompanies NSAID use. Since plasma potassium is tightly regulated by several different effector systems, NSAID-induced hyperkalemia seldom occurs in the absence of other defects in potassium homeostasis. The mechanism of NSAID action is the suppression of prostaglandin-mediated renin release leading to a state of hyporeninemic hypoaldosteronism. Patients at risk are those on drugs that block internal potassium homeostasis (beta blockers, alpha agonists) or drugs that reduce potassium excretion (potassium-sparing diuretics, aldosterone antagonists). Insulin-dependent diabetic patients, especially with renal dysfunction, as well as patients with moderate to severe renal failure (glomerular filtration rate $< 30$ mL/min) are at particularly high risk.

Hyponatremia secondary to a defect in free water clearance is well documented in the use of NSAIDs. Abundant evidence indicates that prostaglandins antagonize the hydro-osmotic effect of antidiuretic hormone. Thus, NSAID use enhances antidiuretic hormone action and promotes water retention. This effect is further accentuated by the effect of NSAIDs to augment medullary tonicity by enhancing the active transport of chloride at the thick ascending limb of the loop of Henle. Restriction of water intake may be necessary in those patients who develop hyponatremia during NSAID use.

## ACUTE INTERSTITIAL NEPHRITIS AND MINIMAL-CHANGE GLOMERULOPATHY

Nonsteroidal anti-inflammatory drugs of all classes have been reported to induce a syndrome of acute interstitial nephritis with or without minimal-change glomerulopathy. This rare syndrome has been reported after 2 to 18 months of NSAID therapy and may be sufficiently severe as to require dialysis support. Most cases are reversible and are characterized pathologically by a mononuclear cell infiltrate of lymphocytes and plasma cells. When there is glomerular involvement, the predominant lesion is epithelial cell podocyte fusion detected by electron microscopy. The most culpable NSAID appears to be fenoprofen, although virtually all NSAIDs have been reported to induce this pathology. Acute interstitial nephritis is probably the most common presentation, followed by combined interstitial and glomerular disease; the least common is minimal-change glomerulopathy. The usual stigmata of an allergic syndrome are absent, such as skin rash, peripheral eosinophilia, and increased immunoglobulin E level, suggesting that the mechanism of action may be related to a reactive noncyclo-oxygenase product of arachidonic acid metabolism. The syndrome is usually reversible by the withdrawal of the offending NSAID. There are no controlled studies supporting the use of corticosteroids to alter the rate or extent of renal recovery.

## NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AND CHRONIC RENAL DISEASE

Despite the well-characterized acute biologic effects of NSAIDs on the kidney, there are no scientifically acceptable data documenting the safety of this class of drugs on renal structure and function when taken chronically. Epidemiologic data show an 8.8 increased relative risk of end-stage renal disease in subjects ingesting 5,000 or more doses of NSAIDs compared with control subjects matched for age. However, these data are flawed by the study design and do not neces-

sarily support a cause and effect relationship.[9] In a better-designed, multicenter, case control study, the risk of chronic renal disease defined as a serum creatinine of ≥1.5 mg/dL was 2.1 (95% confidence interval, 1.1 to 4.1) in daily users of NSAIDs.[10]

The hallmark lesion of analgesic-associated nephropathy is renal papillary necrosis, which can lead to progressive renal failure but also may be present with a well-preserved glomerular filtration rate, making ascertainment of cases by renal function studies alone problematic. In a prospective radiographic study of 259 patients with an intake of 1,000 to 26,000 NSAID doses, papillary necrosis was found in 38 users who took predominately physician-prescribed NSAIDs. Only 65% of these patients had renal functional impairment. Thus, it is clear that long-term use of NSAIDs can cause renal papillary necrosis and renal insufficiency.[11] The frequency of renal papillary necrosis as a primary or contributing cause of end-stage renal disease is unknown because of the infrequent radiographic diagnosis by physicians resulting in misclassification and the insensitivity of renal diagnosis by currently available renal function tests, such as serum creatinine. Furthermore, other known effects of NSAIDs on the kidney, including increased blood pressure and renal hemodynamic changes, could contribute to facilitating progressive renal disease of other etiologies. The experimental production of renal papillary necrosis by NSAIDs is enhanced by caffeine.[12] It is not known whether this is clinically relevant because caffeine intake has not been considered in epidemiologic or other clinical studies.

While there is an extensive package insert documenting the renal consequences of prescription NSAIDs, there are no renal warnings at all on over-the-counter NSAIDs, which are heavily advertised to the public. Thus, patients in high-risk groups or patients with pre-existing kidney disease could be unaware that they have been exposed to these drugs. Case reports and case series document the ability of a variety of chemically unrelated NSAIDs to produce renal papillary necrosis and renal insufficiency.[13-16]

There are other causes of chronic renal failure in patients using prescription or over-the-counter NSAIDs. Although acute renal dysfunction due to NSAIDs is most often reversible, approximately 20% of reported cases have permanent renal failure whether the NSAIDs produced ARF via acute tubular necrosis, acute interstitial nephritis with proteinuria, or simply renal blood flow decreases in high-risk populations.[17]

Irreversible renal failure may also occur in children.[18] Prenatal exposure to indomethacin may lead to severe irreversible renal failure, which is favored by prior stimulation of the renin-angiotensin system. Since these infants are not generally candidates for renal replacement, the consequences of NSAIDs in this setting are not reflected in the end-stage renal disease statistics of the US Renal Data System. In recent series, neonatal renal failure deaths were reported with 150 to 400 mg of indomethacin per day for 2 to 11 weeks during pregnancy.[19-22] Low birth weights and hyperkalemia also have been described in surviving infants.[23]

When NSAIDs are used to reduce proteinuria in nephrosis, permanent renal damage has been reported. Another potential adverse effect of NSAIDs in patients with chronic renal failure includes fatal hyperkalemia from drug–drug interactions with angiotensin-converting enzyme inhibitors, potassium-sparing diuretics, or beta blockers.[24]

Although the population exposure to prescription and nonprescription NSAIDs is large, even the estimated 1% patients with clinically detectable renal dysfunction has important medical and economic implications.[25] The longest period of observation with regard to chronic NSAID usage is 6 to 12 months. In the United States, there are no cross-sectional or prospective studies applied to NSAIDs using the objective criteria for analgesic nephropathy diagnosis proposed by Elseviers and DeBroe,[26] although these criteria have been validated in Europe. In a large general internal medicine practice in which records of analgesic users were surveyed, patients older than 65 years and those with coronary artery disease were at risk of renal impairment with NSAIDs compared with users of acetaminophen. No radiographic data are available.[27]

## SUMMARY

Nonsteroidal anti-inflammatory use in the general population is safe and efficacious when used in therapeutic dosages for a limited period of time. In contrast, patients with pre-existing risk factors are susceptible to potentially life-threat-

ening toxicities, including ARF and serious fluid and electrolyte disorders. Numerous studies have delineated the mechanism(s) by which NSAIDs induce these adverse effects and identify the patients at highest risk (Table 1). The safe use of these agents requires the identification of these risk factors, interventions to ameliorate these risks when possible, and the careful monitoring of renal function and electrolyte concentrations to avoid serious complications.

Renal papillary necrosis and chronic renal insufficiency can occur secondary to prolonged use of prescription and over-the-counter NSAIDs. Neonatal renal failure and renal death may occur from use during pregnancy. While acute renal failure due to NSAIDs occurs in well-defined high-risk patients or under rare idiosyncratic circumstances, renal recovery is incomplete in approximately 20% of reported cases. There are epidemiologic data to support NSAID use as a risk factor for chronic renal dysfunction, even end-stage renal disease, in a cumulative dose-dependent fashion. Despite over-the-counter status, there are no long-term studies of renal structure or function that document the safety of these drugs.

## CONCLUSIONS

1. Use of NSAIDs in the general population is safe and effective when used in therapeutic dosages for a limited period of time.
2. Patients with pre-existing risk factors are susceptible to potentially life-threatening toxicities, including ARF and serious fluid and electrolyte disorders.
3. Renal papillary necrosis and chronic renal failure can occur secondary to prolonged use of prescription and over-the-counter NSAIDs.
4. Neonatal renal failure and renal death may occur from NSAID use during pregnancy.
5. Nonsteroidal anti-inflammatory drug-induced ARF is usually, but not inevitably, reversible.
6. There are no acceptable epidemiologic or clinical data regarding the risk of NSAIDs for chronic renal failure, renal papillary necrosis, or end-stage renal disease.
7. There are no data of NSAIDs' effect on progression of other renal diseases (experimental or clinical).

## RECOMMENDATIONS

1. There should be an explicit label to warn patients taking over-the-counter NSAIDs of potential renal toxicities (similar to that suggested in Am J Kidney Dis 6:4-5, 1985).
2. Design and implement properly controlled studies on the renal and cardiovascular safety of chronic NSAIDs by themselves or in the presence of other known etiologies of renal disease.
3. Combinations of NSAIDs with other analgesics and/or caffeine should be prospectively evaluated for renal safety prior to release.

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# Effects of NSAIDs on the kidney.

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NSAID use is pervasive in our society. Existing NSAIDs pose little risk to patients who tolerate them early during their administration. Among persons with normal renal function who have no other risk factors (dehydration) for an acute hemodynamic effect, there is no risk. However, NSAID administration to susceptible persons may cause decrements in renal plasma flow and glomerular filtration rate within hours. This acute hemodynamic effect is the most common renal syndrome caused by NSAIDs. With careful monitoring, this effect is readily detected with routine clinical laboratory tests (serum creatinine and/or blood urea nitrogen concentrations). However, patients who continue administration of NSAIDs in this setting risk acute tubular necrosis and permanent damage to the kidney. Newer NSAIDs that selectively inhibit cyclooxygenase-2: cyclooxygenase-1 ratio may provide a more favorable risk profile for patients who cannot tolerate existing drugs.

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